Genetic engineering of cell wall melanin biosynthesis in the emerging human pathogen *Lomentospora prolificans*

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Abstract

The dematiaceous (melanised) fungus *Lomentospora* (*Scedosporium*) *prolificans* is a life-threatening opportunistic pathogen of immunocompromised humans, resistant to anti-fungal drugs. Melanin has been shown to protect human pathogenic fungi against antifungal drugs, oxidative killing and environmental stresses. To determine the protective role of melanin in *L. prolificans* to oxidative killing (H_2O_2), UV radiation and the polyene anti-fungal drug amphotericin B, targeted gene disruption was used to generate mutants of the pathogen lacking the dihydroxynaphthalene (DHN)-melanin biosynthetic enzymes polyketide synthase (PKS1), tetrahydroxynapthalene reductase (4HNR) and scytalone dehydratase (SCD1). Infectious propagules (spores) of the wild-type strain 3.1 were black/brown, whereas spores of the PKS-deficient mutant Δ*Lppks1*::*hph* were white. Complementation of the albino mutant Δ*Lppks1*::*hph* restored the black-brown spore pigmentation, while the 4HNR-deficient mutant Δ*Lp4hnr*::*hph* and SCD-deficient mutant Δ*Lpscd1*::*hph* both produced orange-yellow spores. The mutants Δ*Lppks1*::*hph* and Δ*Lp4hnr*::*hph* showed significant reductions in spore survival following H_2O_2 treatment, while spores of Δ*Lpscd1*::*hph* and the Δ*Lppks1*::*hph* complemented strain Δ*Lppks1*::*hph*:PKS showed spore survivals similar to strain 3.1. Spores of the mutants Δ*Lp4hnr*::*hph* and Δ*Lpscd1*::*hph* and complemented strain Δ*Lppks1*::*hph*:PKS showed spore survivals similar to 3.1 following exposure to UV radiation, but survival of Δ*Lppks1*::*hph* spores was significantly reduced compared to the wild-type strain. Strain 3.1 and mutants Δ*Lp4hnr*::*hph* and Δ*Lppks1*::*hph*:PKS were resistant to amphotericin B while, paradoxically, the PKS1- and SCD1-deficient mutants showed significant increases in growth in the presence of the antifungal drug. Melanin was shown to play no role in the protection of the pathogen from immune cell recognition and killing by alveolar macrophages, with similar degrees of engulfment, and spore viabilities, of mutant and wild-type strains after phagocytosis. Contrary to expectations, the albino PKS-deficient mutant was significantly more virulent than the melanised wild-type strain during pathogenicity studies in the invertebrate infection model *Galleria mellonella*, with levels of virulence restored to near wild-type levels in the complemented strain Δ*Lppks1*::*hph*:PKS. Taken together, the results presented in this thesis show that melanin protects *L. prolificans* from UV radiation and from oxidative killing by H_2O_2, consistent with its survival in extreme environmental habitats. However, melanin was

not found to play a role in the resistance of the pathogen to the antifungal drug amphotericin B, to protect the fungus from immune cell recognition or killing by alveolar macrophages, or to its pathogenicity.

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1. General Introduction

1.1 Fungi and human disease

Fungi are eukaryotic heterotrophic organisms that reside in different terrestrial and aquatic environments (Webster & Weber, 2007; Horgan & Murphy, 2011), and have commensal, parasitic and saprotrophic lifestyles (Horgan & Murphy, 2011). Fungi have many beneficial traits which are exploited by humans (Al-Fakih, 2014), such as the recycling of organic materials (Artigas, *et al*., 2004), the production of natural products such as pharmaceuticals (e.g. lovastatin and antibiotics) (Hendrickson, *et al*., 1999), and enzymes used in food production and industrial processing (Lange, 2014).

However, fungi constitute a serious threat to global food security, animal welfare and human health since numerous species distributed across the four main phyla of fungi (chytridiomycetes, zygomycetes, ascomycetes and basidiomycetes) are able to infect crop plants, animals and humans, causing devastating crop losses and life-threatening mycotic infections (Bennett & Klich, 2003; Dean, et al., 2012; Khan, *et al*., 2010; Kheder, *et al*., 2012).

Human pathogenic fungi are often environmental organisms such as *Aspergillus fumigatus* and *Fusarium solani* or are part of our normal skin or mucosal flora such as *Candida albicans* and *Geotrichum candidium* (Thomakos , *et al*., 1998; Deepa, *et al*., 2014). Kendrick (2000) classified human pathogenic fungi into three main categories: dermatophytes that cause cutaneous (skin) infections (e.g. *Microsporum audouinii*); dimorphic pathogens such as *Histoplasma capsulatum*; and opportunistic fungi that cause infections in patients with impaired immune systems (Kendrick, 2000). Mycoses are classified as superficial, cutaneous, sub-cutaneous, and systemic (Figure 1.1) (Walsh & Dixon, 1996), the latter can lead to disseminated infections spreading to multiple sites within the susceptible host (Rabodonirina , *et al*., 1994). Even with a fully functioning immune system, seemingly healthy individuals can acquire serious infections from environmental moulds such as *Scedosporium apiospermum* and *Lomentospora prolificans*, yeasts such as *Cryptococcus neoformans,* and endemic fungi such as *Coccidioides immitis* (Cooley, *et al*., 2007; Suchitha, *et al*., 2012; Chiam, *et al*., 2013).

Invasive fungal infections (IFIs) are associated with high rates of morbidity and mortality (Abu-Elteen & Hamad, 2012), with *Candida* and *Aspergillus* species

responsible for the majority of life-threatening infections (Lamagni, *et al*., 2001). However, other fungi have emerged as serious human pathogens over the past decade including species in the *Pseudallescheria*/*Scedosporium* complex and *Fusarium* species, which similarly cause high rates of mortality in immunocompromised patients (Lamaris, *et al*., 2006; Ravikant, *et al*., 2015). Consequently, it is important to recognise the threat that fungi pose to human health in the same way we consider bacteria, viruses and parasites (Netea & Brown, 2012).

Two major risk factors for IFIs are identifiable. The first is the major advances in clinical medicine that now allow solid organ and haematopoietic stem cell transplantation (HSCT) as routine procedures and which, when coupled to highly effective immunosuppression and aggressive anti-cancer regimens, have dramatically improved the survival rates of, for example, cancer patients. Ironically, however, the impaired immunity in these patients that prevents graft rejection, increases substantially the risk of acquiring fatal fungal infections (Richardson, 2005). The second risk factor emerged in the 1980s with the appearance of Acquired Immune Deficiency (AIDS), to the extent that mycoses caused by *Pneumocystis carinii* (Pneumocystis pneumonia) and *Talaromyces* (*Penicillium*) *marneffei* (penicilliosis) are AIDS-defining diseases (Miller, *et al*., 1996; Wong & Wong, 2011; Kozel & Wickes, 2014).

1.2 The clinical importance of dematiaceous (melanised) fungi

Morphologically, fungi are characterised as pigmented and non-pigmented (hyaline hyphae), and depends on their ability to synthesize melanin (Ajello, 1986). Many terms have been used to described melanised fungi (Pappagianis & Ajello, 1994), and currently pigmented species are referred to as dematiaceous (Revankar & Sutton, 2010).

Dematiaceous fungi are recovered from diverse habitats including air, soil, water, plants, sewage, manure, and pigeon droppings (Latgé, 1999; Panackal & Marr, 2004; Soltani, *et al*., 2013). Some species inhabit extreme environments such as the halophilic (salt-loving) species *Hortaea werneckii* (Kogej, *et al*., 2004). Certain dematiaceous fungi are etiological agents of human disease causing

phaeohyphomycosis, eumycetoma, chromoblastomycosis and fungemia (Hoffmann, *et al*., 2011).

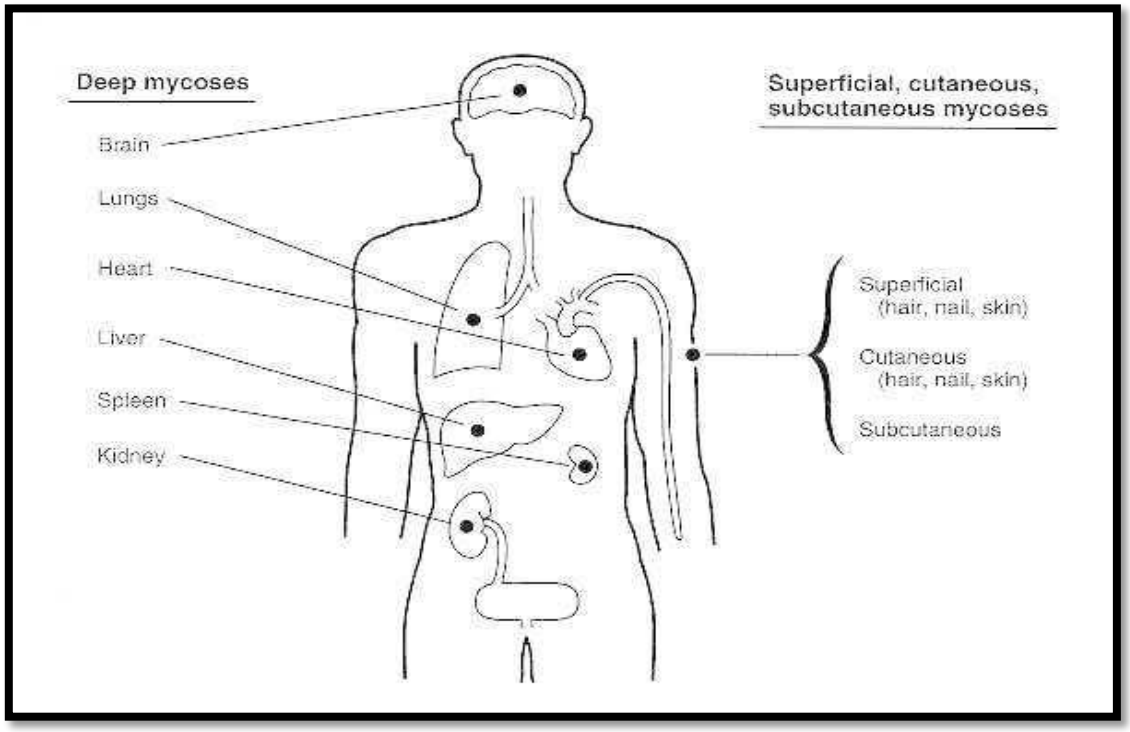

Figure 1.1: Potential sites of fungal infections in the human body, from superficial skin infections to disseminated infections of solid organs. Figure taken from (Walsh & Dixon, 1996).

Mycetoma, a chronic infection of skin and subcutaneous tissues is reported mainly mostly in tropical regions such as Sudan, India and Mexico (Ameen & Arenas, 2008; Bonifaz, *et al*., 2014; Fahal, *et al*., 2015; Rit, *et al*., 2015). The principle agents of the disease are actinomycete bacteria (mycetoma) or fungi (eumycetoma) (Ameen & Arenas, 2008). The most important fungal agents are *Madurella mycetomatis and Pseudallescheria boydii* (Horré , *et al*., 2002; Brufman, *et al*., 2015). Other species include *Exophiala jeanselmei, Cylindrocarpon* spp., *Leptosphaeria tompkinsii, Scedosporium apiospermum, Aspergillus* spp. and *Fusarium solani* (Hemashettar, *et al*., 2000; Yera, *et al*., 2003; Capoor, *et al*., 2007; Machmachi , *et al*., 2011; Oliveira, *et al*., 2013; Hopps, *et al*., 2014). The symptoms caused by these organisms are very similar (Welsh, *et al*., 2014), with chronic infections of the cutaneous and

subcutaneous tissues containing characteristic ‘grains’ within mycetoma lesions (Fahal, *et al*., 2015). Eumycetomas produce white or black grains depending on the causative agents (Hemashettar, *et al*., 2000; Machmachi , *et al*., 2011). The most common site of infection is the foot, followed by the head and neck, chest wall, back, abdominal wall or perineum (Fahal, *et al*., 2015). Surgical debridement of the lesion is generally needed prior to treatment with an appropriate antifungal drug (Hjira, *et al*., 2015).

Chromoblastomycosis (CBM) is a chronic cutaneous and subcutaneous infection caused by dematiaceous fungi such as *Fonsecaea pedrosoi*, *Cladophialophora carrionii, Exophiala dermatitidis and Phialophora verrucosa* (Hofmann, *et al*., 2005; Kim, *et al*., 2011; Pradeepkumar & Joseph, 2011; Krzyściak, *et al*., 2014). The disease in restricted top moist tropical and subtropical area such as in Mexico, Indian (Bobba, 2014; Dashatwar , *et al*., 2015), Brazil (Correia, *et al*., 2010), and Cuba (Badali, *et al*., 2013). Infection is typically by skin puncture followed by development of a painless solitary lesion and then *i*tchy nodules and plaques with erythematous and verrucous edges (Ezughah, *et al*., 2003; Badali, *et al*., 2013). Single lesions then spread to form multiple lesions (Troncoso *&* Bava, 2009; Bobba, 2014). CBM is distinguished histologically from phaeohyphomycosis by the presence of sclerotic bodies (muriform cells) within the lesion (McGinnis, 1983). Most cases of the disease are found in rural workers (particularly males) with bare feet (Pradhan, *et al*., 2007; Queiroz-Telles , *et al*., 2009), with infections in the lower limbs, followed by upper limbs, ankles, and face (Correia, *et al*., 2010). As with mycetomas, treatment typically requires surgical removal of lesions, chemotherapy (such as itraconazole) or combination therapy (Huang, *et al*., 2008; Queiroz-Telles , *et al*., 2009).

Ajello and co-workers introduced a new term (phaeohyphomycosis) to describe infections caused by the dematiaceous fungus *Phialophora parasitica* (Ajello, *et al*., 1974). The term hyalohyphomycosis describes infections caused by non-pigmented (hyaline) fungi (Cocuroccia, *et al*., 2003). The term phaeohyphomycosis now incorporates a wide range of localised and disseminated diseases caused by different dematiaceous fungi (Revankar, 2010; Seyedmousavi, *et al*., 2013). Rare cutaneous, subcutaneous and disseminated cases have been reported in healthy individuals, whereas life-threatening infections are found in immunocompromised patients

(Revankar, *et al*., 2004; Ge, *et al*., 2011; Mudholkar, *et al*., 2011; Binesh, *et al*., 2016). A wide spectrum of infectious agents cause phaeohyphomycosis including members of the *Pseudallescheria*/*Scedosporium* complex, *Wangiella dermatitidis, Cladophialophora bantiana*, *Alternaria alternata, and Lomentospora prolificans* (Simarro , *et al*., 2001; Revankar, *et al*., 2004; Gomes, *et al*., 2011; Sla´dekova, *et al*., 2014).

1.3 *Lomentospora prolificans*

Lomentospora prolificans (formerly *Scedosporium prolificans*) is a dematiaceous ascomycete fungus (Cortez, *et al*., 2008), and is related phylogenetically to members of the *Pseudallescheria*/*Scedosporium* complex (Lackner, *et al*., 2014). It is a pathogen capable of infecting both immunocompromised and seemingly immunocompetent individuals (Steinbach, *et al*., 2003 ; Chiam, *et al*., 2013), as well as wild and domesticated animals (Haynes, *et al*., 2012; Swerczek, *et al*., 2001). As with members of the *Pseudallescheria*/*Scedosporium* complex, the incidence of diseases caused by this emerging pathogen *L. prolificans* has increased recently (Bouchara, *et al*., 2009). The fungus has been recovered from soil (Hennebert & Desai, 1974) and from pot plants (Summerbell *et al*., 1989; Gosbell *et al*., 1999), but the wider distribution of the pathogen is not well known (Thornton & Wills, 2015a). It was first described as an agent of osteomyelitis in 1984 and was called *Scedosporium inflatum* (Malloch & Salkin, 1984). Since then, its nomenclature has been revised to *Scedosporium prolificans* and, more recently, to *L. prolificans* (Hennebert & Desai, 1974; Malloch & Salkin, 1984; Guého & Hoog, 1991; Lackner, *et al*., 2014).

L. prolificans is known as dematiaceous fungi owing to synthesize DHN-melanin, therefore colonies of *L. prolificans* displayed grey colour on oat meal agar media plates (Al-Laaeiby, *et al*., 2016), and olive-gray to black colonies onto Sabouraud dextrose agar (SDA) (Elsayed, *et al*., 1999). Cortez *et al*., 2008 described *L. prolificans* as an anamorphic fungus that has septate hyphe containing conidiophores carrying single or groups of the oval spores. Production of conidiophores, which characterised by swollen bases, and absence of graphium are distinguished features than *Scedosporium apiospermum* (Cortez, *et al*., 2008). Growth of *L. prolificans* is inhibited in the media supplemented with cycloheximide, hygromycin b and sulfonyl urea, in

addition to their inability to assimilate sucrose, ribitol, xylitol and L-arabinitol (de Hoog, *et al*., 1994; Ruiz-Díez & Joaquín, 1999 ; Al-Laaeiby, *et al*., 2016)

Nosocomial and community acquired infections by *L. prolificans* have been reported in the UK, Spain, Germany and Australia (Berenguer *et al*., 1997: Cooley *et al*., 2007: Sedlacek *et al*., 2015). Infections are acquired by inhalation of spores, wound contamination (Steinbach, *et al*., 2003 ; Bhagavatula, *et al*., 2014), or colonization (Tintelnot , *et al*., 2009). Patients with acute myeloid leukaemia (Chiam, *et al*., 2013), immunodeficiency diseases such as AIDS (Nenoff , *et al*., 1996), bone marrow and lung transplants, stem cell transplantation, myelodysplastic syndrome (MDS) and hematologic malignancy are at risk of infection with *L. prolificans* (Cooley, *et al*., 2007; Tintelnot , *et al*., 2009). Localized and non-lethal infections have been reported in immunocompetent individuals, whereas in immunocompromised patients invasive, disseminated infections are typically fatal (Abu-Elteen & Hamad, 2012) resulting from endophthalmitis, central nervous system infection, osteomyelitis, endocarditis, fungaemia, and keratitis (Steinbach, *et al*., 2003 ; Tintelnot , *et al*., 2009; Chiam, *et al*., 2013; Ochi, *et al*., 2015; Chandel, *et al*., 2011; Trubiano , *et al*., 2014). A reason for the high rates of mortality following infections by *L. prolificans* is its intrinsic resistance to antifungal drugs such as the azoles and amphotericin B (Gosbell *et al*., 1999; Cortez *et al*., 2008). Combination therapy has led to successful resolution of brain abscesses in a chronic granulomatous disease (CGD) patient (Bhat, *et al*., 2008), but combination therapy and surgical debridement is most often needed for full recovery (Howden, *et al*., 2003). Azole specifically voriconazole is a candidate antifungal drug to be more effective against *L. prolificans* alone or in combination with terbinafine (Meletiadis, *et al*., 2003). Morphological, cell wall carbohydrate constitute and proteomes shifts were companied *L. prolificans* in response to voriconazole treatment (Rodríguez, *et al*., 2017).

1.4 The fungal cell wall

The cell wall is a dynamic and elastic layer surrounding the cell membrane and is remodelled during growth, development or stress (Cabib, *et al*., 2001). The principal functions of the fungal cell wall are cell viability, growth, shape, physical protection, adherence to surfaces, osmotic stability, protection against environmental stresses

and communication with the surrounding environment (Cabib, *et al*., 1997; de Nobel , *et al*., 2000). The basic structure of the fungal cell wall comprises two layers, an inner fibrous (polysaccharide) layer composed of β-1,3- and β-1,6-linked glucan linked to chitin, which is cross-linked to an outer matrix consisting of mannoprotein (Latgé, 2010). The synthesis and assembly of the cell wall in filamentous fungi occurs at the hyphal apex, whereas in yeasts it is generated and assembled at bud tips (Osherov & Yarden, 2010). The main three components of the fungal cell wall (β-glucan, chitin and glycoprotein) are combined covalently by cell wall cross-linking enzymes (Free, 2013). In dematiaceous species such as *L. prolificans*, melanin is also a cell wall component (Pihet, *et al*., 2009).

1.5 Melanin

The word melanin derives from the ancient Greek word *melanos*, and was first coined by the Swedish chemist Berzelius in 1840 (Solano, 2014). Evidence suggests that melanin is ancient, with similar pigments discovered in the remains of dinosaurs, and which are used as a tool for studying evolutionary development (Zhang, *et al*., 2010; Solano, 2014). Today, these pigments are widely distributed among lifeforms including prokaryotic and eukaryotic organisms (Nicolaus, *et al*., 1964; Banerjee, *et al*., 2014).

Melanin refers to black pigments produced by the oxidation and polymerization of phenolic compounds of high molecular weight (Jacobson, 2000). They are amorphous, insoluble, negatively charged, hydrophobic polymers that can be bleached by oxidative agents (hypochlorite or H_2O_2), but are resistant to hydrolytic enzymes (Nicolaus, *et al*., 1964; Butler, *et al*., 1989; Nosanchuk & Casadevall, 2006).

The physical and chemical techniques that can be used to identify melanins are limited (Nosanchuk & Casadevall , 2003). Chemical degradation is the most popular method for melanin isolation (Piattelli, *et al*., 1965; Arnau & Bore, 1981), which results in the production of hollowed structures called melanin ghosts, discernible under transmission and scanning electron microscopy (Wang, *et al*., 1996). Examination of the melanin ghost surface by atomic force microscopy (AFM), and scanning electron microscopy, reveals that the spherical shape of melanin ghost consists of irregular, detached granules 40-130 nm in diameter, arranged in concentric layers as shown in Figure 1.2 (Eisenman, *et al*., 2005).

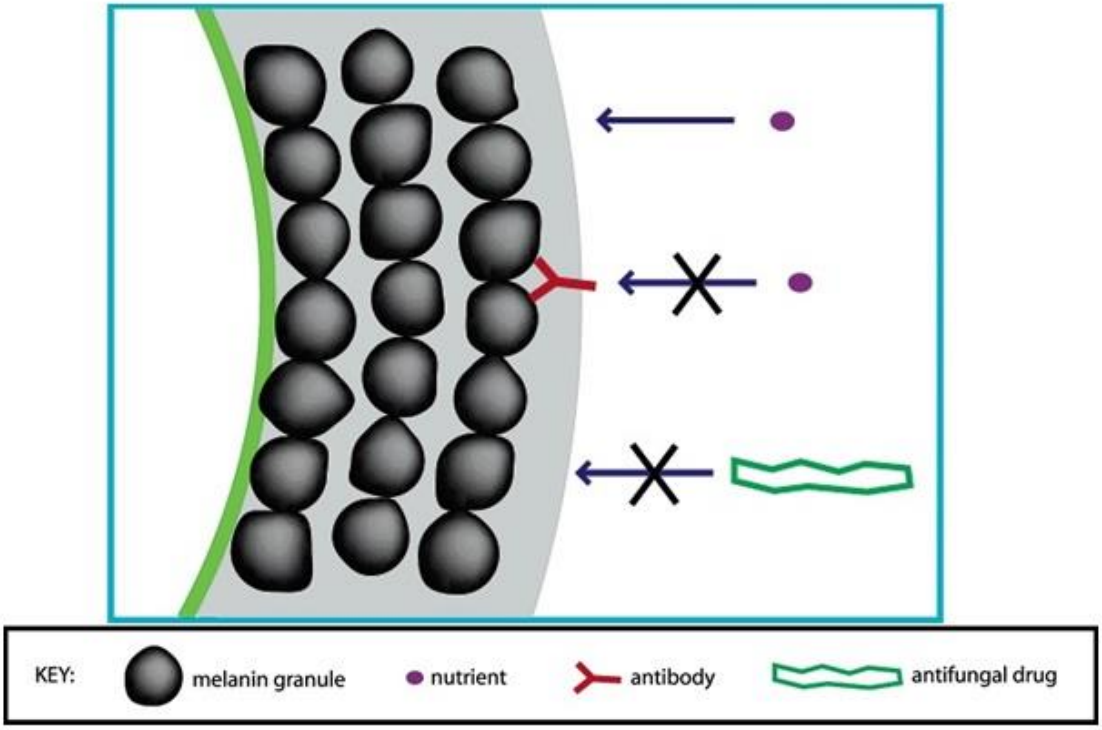

Figure 1.2: Schematic representation of a cross section of a melanin ghost from the human pathogenic yeast *Cryptococcus neoformans*. Figure taken from Eisenman and co-workers (2005). Microscopy reveals a multi-layered structure containing granular particles. This particulate structure allows movement of nutrients into the cell, but prevents entry of larger molecules such as antifungal drugs and antibodies.

The particulate multi-layered structure allows for small and larger pores with diameters of approximately 1-4 nm and 30 nm, respectively (Eisenman, *et al*., 2005). Jacobson and Ikeda (2005) demonstrated differences in the porosities of the cell walls of melanised and non-melanised *Cryptococcus neoformans* strains due to differences in pore sizes of ~4 nm and 16 nm, respectively (Jacobson & Ikeda, 2005).

While all studies of melanin in fungi have shown it to be in the outer layers of the cell, its localisation differs across species. In *Cryptococcus neoformans*, transmission electron microscopy reveals an electron dense layer indicative of melanin in the outer regions of the plasma membrane (Wang, *et al*., 1995; Nosanchuk & Casadevall , 2003), while in other fungal pathogens including *Paracoccidioides brasiliensis,*

Histoplasma capsulatum, Candida albicans, Aspergillus spp., Sporothrix schenckii, Fonsecaea pedrosoi, and *Coccidioides* spp., melanin pigments are found in the cell wall or on its surface (Nosanchuk, *et al*., 2015).

1.5.1 Types of melanin and their biosynthesis

A range of melanins are synthesized using a number of different pathways and chemical precursors (Solano, 2014). Eumelanins and pheomelanins are produced in the hair and skin of the mammals (Thody, *et al*., 1991), while plants use the enzyme catechol oxidase to synthesize another type of melanin lacking nitrogen, known as catechol-melanin (allo-melanin) (Solano, 2014). Bacteria use homogentisic acid to synthesize pyomelanin (Kotob, *et al*., 1995).

Two common types of melanin are found in fungi, namely 1,8-dihydroxynaphthalene (DNH) melanin and L-1,3-Dihydroxyphenylalanine melanin (L-DOPA) (Eisenman & Casadevall, 2012). DHN-melanin is produced by the human pathogens *Lomentospora prolificans*, *Penicillium marneffei*, *Aspergillus fumigatus*, *Wangiella dermatitidis*, *Sporothrix schenckii, and Torula coralline*, and the plant pathogens *Colletotrichum lagenarium*, and *Gaeumannomyces graminis* (Romero-Martinez, *et al*., 2000 ; Woo, *et al*., 2010; Bayry, *et al*., 2014; Thornton, *et al*., 2015b; Tsuji, *et al*., 2001; Schnitzler, *et al*., 1999 ; Lee, *et al*., 2003 ; Caesar-Tonthat, *et al*., 1995).

Figure 1.3A illustrates the stages of DHN melanin biosynthesis in fungi (Eisenman & Casadevall, 2012), in which the enzyme polyketide synthase catalyzes the fusion and cyclisation of five units of the precursors acetyl CoA or malonyl CoA (which are synthesized endogenously) to produce 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) (Takano, *et al*., 1995; Schneiter, *et al*., 1996 ; Fujii, *et al*., 1999; Webster & Weber, 2007). 1,3,6,8-THN is then reduced by tetrahydroxynaphthalene reductase (4HNR) to scytalone (Thompson, *et al*., 2000). Scytalone dehydratase (SCD) then dehydrates scytalone to 1,3,8-trihydoxynaphthalene (Bell & Wheeler, 1986). Further reduction and dehydration steps yield vermelone and 1,8-dihydroxynaphthalene (DHN) respectively (Thompson, *et al*., 2000). Melanin is finally produced after a series of polymerizations of 1,8-dihydroxynaphthalene (DHN) catalyzed by the enzyme laccase (Webster & Weber, 2007).

Figure 1.3B illustrates the biosynthesis of L-3,4-dihydroxyphenylalanine (L-DOPA) melanin which is found in many fungal species including *Cryptococcus neoformans*, *Aspergillus nidulans*, *Penicillium marneffei*, and *Candida albicans* (Goncalves, *et al*., 2012; Winter, *et al*., 1999; Wang, *et al*., 1996 ; Liu, *et al*., 2014; Morris-Jones, *et al*., 2005). The precursors of L-DOPA melanin are generally acquired exogenously (Kwon-Chung, *et al*., 1983), with indolic compounds possessing hydroxyl or amine group at the phenyl ring used as substrates for the enzyme phenol oxidase (Kwon-Chung, *et al*., 1983). If tyrosine is used as a precursor, it is first converted to L-dihydroxyphenylalanine by the enzyme tyrosinase, and then oxidised to dopaquinone (Eisenman & Casadevall, 2012). Both tyrosinase and laccase are phenol oxidases, requiring copper as a co-factor (Langfelder, *et al*., 2003). The intermediate product dopaquinone comprises leucodopachrome in the absence of thiol, which later oxidizes forming dopachrome (Langfelder, *et al*., 2003). Dopachrome hydroxylates and carboxylates forming dihydroxyindoles, with L-DOPA melanin forming after the polymerization of the dihydroxyindoles (Langfelder, *et al*., 2003).

Some fungal species are able to produce more than one type of melanin. For instance, *Aspergillus fumigatus* produce pyomelanin, utilizing tyrosine as the precursor (Schmaler-Ripcke, *et al*., 2009), as well as DHN-melanin (Bayry, *et al*., 2014). *Sporothrix schenckii* similarly produces DHN-melanin and L-DOPA (Almeida-Paes, *et al*., 2009).

Almeida-Paes *et al.* (2009) determined the melanin producing capacity of *Sporothrix schenckii* under different growth conditions, and showed its biosynthesis was governed by pH, temperature and nutrients (Almeida-Paes, *et al*., 2009). Copper availability also affects pigmentation by contributing to laccase activity (Griffith, *et al*., 2007), with expression of the laccase encoding gene *LAC1* induced by copper (Jiang, *et al*., 2009), and the copper-responsive transcription factor *CUF1* regulating the expression of *LAC1* (Jiang, *et al*., 2009).

In higher animals, melanocytes synthesize melanin inside organelles called melanosomes (Van Den Bossche, *et al*., 2006) which isolate toxic intermediate compounds generated during melanin biosynthesis (Hearing, 2005). Melanosomes have also been found in a number of fungi including *Candida albicans* and *Fonsecaea*

pedrosoi (Franzen, *et al*., 2008; Walker, *et al*., 2010), and contain a fibrillar matrix where pigments and iron are deposited during maturation of melanin and its delivery to the cell wall (Franzen, *et al*., 2008). In a similar way, melanin is synthesized within vesicles in *Cryptococcus neoformans* (Eisenman, *et al*., 2009), and then assembles with lipid in the cell wall (Eisenman, *et al*., 2009).

1.5.2 Expression of melanin biosynthetic genes

1.5.2.1 L-DOPA melanin

Melanin production in fungi is induced by environmental factors such as abundance of di-phenolic substrates or glucose deprivation (Nurudeen & Ahearn, 1979). Alspaugh and co-workers demonstrated the role of *GPA1* (encoding a G-protein α-subunit) in regulating melanin biosynthesis in *Cryptococcus neoformans* (Alspaugh, *et al*., 1997). *GPA1*-deficient mutants failed to melanise and to express phenol oxidases in the presence of melanin-inducing substrates and under glucose deprivation, in contrast to the wild-type strain and a complemented mutant (Alspaugh, *et al*., 1997). Addition of exogenous cAMP (which allows Gα protein production via adenylate cyclase), restored melanin production in the *GPA1*-deficient mutant to levels similar to that of the wild-type and complemented strains (Alspaugh, *et al*., 1997). Microarray and northern blot analysis showed that the Gα protein Gpa1 regulates the transcription of the genes *LAC1* and *LAC2* (Pukkila-Worley, *et al*., 2005).

The genes *LAC1* and *LAC2* encode the enzyme laccase. Laccase is a polyphenol oxidase oxidising different a range of phenolic compounds including hydroquinone and catechol (Thurston , 1994). Nagai *et al* (2003) purified two laccases (Lcc1 and Lcc2) from the fruiting bodies (mushrooms) of *Lentinula edodes* during post-harvest storage (Nagai, *et al*., 2003). Laccase enzymes are also involved in lignin degradation (Kawai, *et al*., 1988) in addition to their physiological functions in the fruiting bodies (Suguimoto, *et al*., 2001), and in fungal pathogenesis (Zhu, *et al*., 2001). However, they appear to play little role in resistance to oxidative stresses since enzyme-deficient mutants, for example *Aspergillus fumigatus* mutants with deletions in the laccase-encoding gene *arb2*, show similar levels of sensitivity to H_2O_2 as wild-type strains (Sugareva, *et al*., 2006).

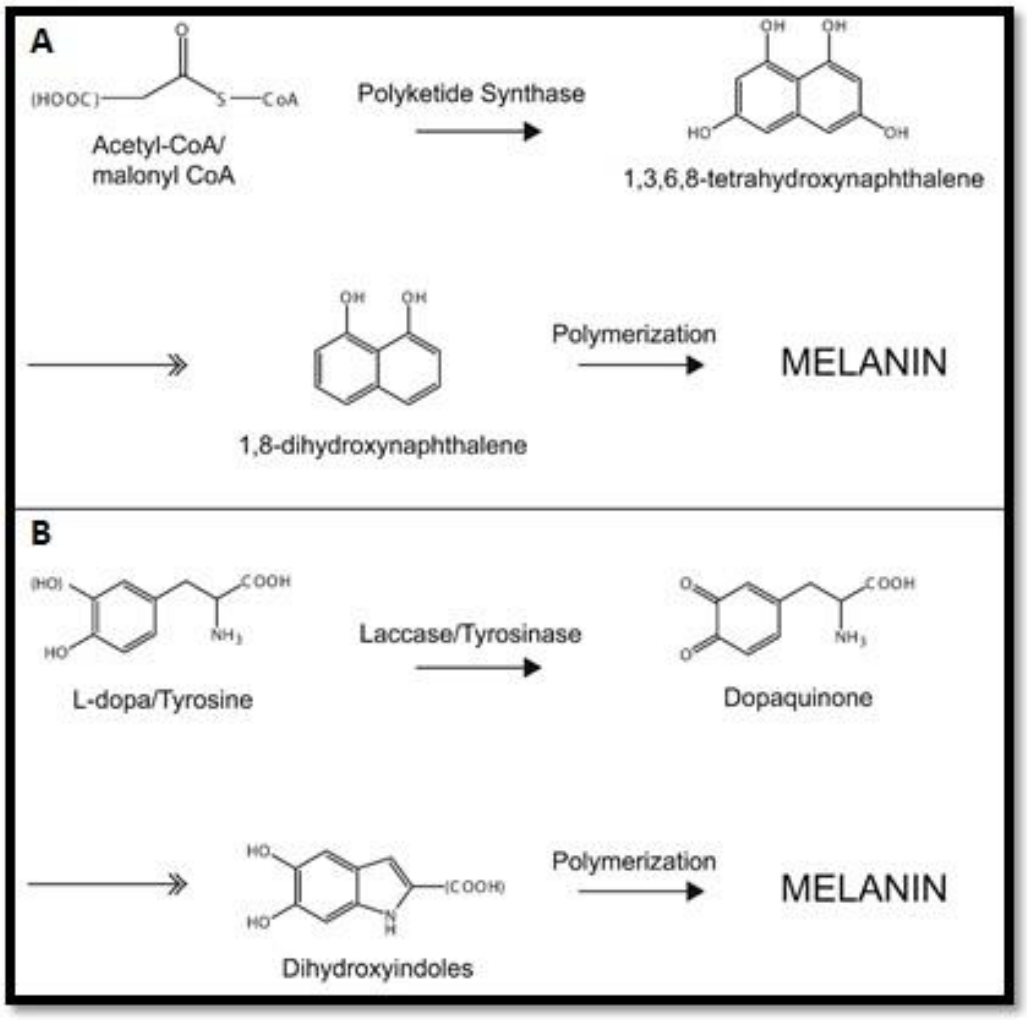

Figure 1.3: Melanin biosynthesis pathways in fungi. A) DHN-melanin biosynthesis pathway. The precursors acetyl-CoA or malonyl-CoA are converted to tetrahydroxynaphthalene by polyketide synthase, with subsequent steps leading to dihydroxynaphthalene formation. Further polymerization yields functional DHN-melanin. B) L-DOPA pathway of eumelanin biosynthesis. The exogenous precursors tyrosine or L-DOPA are converted to dopaquinone by laccase or tyrosinase, with spontaneous reactions leading to dihydroxyindole formation. Subsequent polymerisation leads to the production of eumelanin Figure taken from Eisenman *et al*., 2012.

1.5.2.2 DHN melanin

In plant-pathogenic fungi such as *Magnaporthe grisea* and *Colletotrichum lagenarium*, DHN-melanin is critical to the correct functioning of the appressorium, a specialised infection structure used to penetrate the leaf surface (Talbot, 2003). Kubo and co-workers demonstrated the temperature sensitivity of polyketide synthase activity and DHN-melanin biosynthesis in the appressorium of *Colletotrichum lagenarium* (Kubo, *et al*., 1984). Furthermore, studies of three melanin biosynthesis genes in differentiated and undifferentiated conidia of the fungus showed that gene transcription begins after one hour of incubation of spores in water, with *THR1* (encoding 1,3,8-trihydroxynaphthalene reductase) transcription surprisingly occurring first followed by *PKS1* (encoding polyketide synthase) and then the scytalone dehydratase-encoding gene *SCD1* (Takano, *et al*., 1997). Melanisation of the appressorium is further regulated post-transcriptionally (Kubo, *et al*., 1998). *Colletotrichum* melanin regulation (*CMR1*) gene encodes a transcription factor that regulates the transcription of *THR1* and *SCD1* genes in *Colletotrichum lagenarium* during mycelial melanisation (Tsuji, *et al*., 2000). Mutant lacking the *CMR1* gene display reddish brown colonies due to the accumulation of the melanin intermediate scytalone (Tsuji, *et al*., 2000). In the plant pathogen *Alternaria alternata*, the gene *CMRA*, which encodes a transcription factor, is located within a gene cluster between polyketide synthase (*pksA*) and 1,3,8-THN reductase (*brm2*), and regulates the three genes *pksA, brm2* and *brm1* involved in melanin synthesis (Fetzner, *et al*., 2014).

Thompson and co-workers identified two reductase enzymes in the rice blast pathogen *Magnaporthe grisea* (Thompson, *et al*., 2000). The enzyme encoded by gene *3HN* is able to carry out two reduction steps when added to mutants lacking the gene encoding tetrahydroxynaphthalene reductase, producing the melanin intermediate scytalone (Thompson, *et al*., 2000). The plant pathogenic fungus *Bipolaris oryzae* possesses genes *T4HR1* and *THR1* encoding 1,3,6,8-tetrahydroxynaphthalene reductase and 1,3,8-trihydroxynaphthalene reductase, respectively (Kihara, *et al*., 2004 a; Tanaka, *et al*., 2015). Analysis of the *T4HR1* gene shows that it encodes a protein of 268 amino acids (Tanaka, *et al*., 2015). Homology to counterpart genes in other species (*Magnaporthe grisea*, *Cochliobolus heterostrophus*, *Sordaria macrospora* and *Sordaria macrospora*) has been shown (Tanaka, *et al*., 2015).

1.5.3 Contribution of melanin to the pathogenicity and survival of fungi

Different techniques have been used to generate melanin deficient mutants including physical (UV radiation) or molecular genetic (targeted gene disruption). Regardless of the method used, most studies demonstrate reductions in virulence consequent with loss of melanisation (Jahn, *et al*., 1997; Tsai, *et al*., 1998; Romero-Martinez, *et al*., 2000). This loss of melanisation also typically alters the physical integrity of the fungal cell wall (Pihet, *et al*., 2009).

Most research on the role of melanin to the pathogenicity and survival of human pathogenic fungi has been conducted in the human pathogenic yeast *Cryptococcus*, where both pigmentation and polysaccharide capsule formation have been shown to be important pathogenicity factors (Kwon-Chung & Rhodes, 1986; Karkowska-Kuleta, *et al*., 2009). The negatively charged polysaccharide capsule (Nosanchuk & Casadevall, 1997) shields the yeast cell from phagocytosis and killing by macrophages (Vecchiarelli, *et al*., 1994 ; Buchanan & Murphy, 1998), as well as from environmental predation (Araujo, *et al*., 2012). Molecular genetic studies have shown that capsule-deficient mutants of *Cryptococcus neoformans* are avirulent in an animal model of cryptococcosis (Chang & Kwon-Chung, 1994). Similarly, melanin-deficient strains of the fungus exhibit reduced pathogenicity *in vivo* (Rhodes *et al*., 1982).

Melanin confers several survival advantages to pathogenic fungi (Taborda, *et al*., 2008). The interaction between the two virulence factors of capsule formation and melanisation in *Cryptoccocus* has been studied using melanised, non-capsule-forming, strains, and non-melanized strains, with melanized cells showing recalcitrance to environmental predation (Steenbergen, *et al*., 2001). Melanin also inhibits phagocytosis during cellular (macrophage-mediated) immunity (Schnitzler, *et al*., 1999 ; da Silva, *et al*., 2006; Woo, *et al*., 2010), and reduces oxidative damage to the fungus by scavenging free radicals and reactive oxygen species (Revankar, 2006). However, it triggers the humoral immune response by stimulating antibody production (Nosanchuk, *et al*., 1998; Alviano, *et al*., 2004). Rosas and co-workers (Rosas *et al*., 2000) demonstrated binding of the anti-fungal drug Amphotericin B to melanin, while Rhodes *et al*. (1982) compared its fungistatic effects on melaninsed and non-melanised strains of *Cryptococcus neoformans in vivo*. Higher rates of mortality were found with the melanised strains.

Melanin has also been shown to provide protection to environmental stresses, such as UV light (Brenner & Hearing, 2008) and extremes of temperature (Rosas & Casadevall, 1997), and interacts with metals in the environment (Fogarty & Tobin, 1996). In addition, melanin has been shown to protect fungi against damaging radionuclides (Dadachova, *et al*., 2008).

1.6 Aims and objectives of this study

In this thesis, I set out to investigate the role of melanin in the pathogenicity and survival of the dematiaceous fungus *Lomentospora prolificans*, an emerging pathogen of humans which causes life-threatening infections in immuno-compromised patients. To this end, I describe the use of reverse genetics to disrupt melanin biosynthesis in the fungus by targeted deletion of the genes *PKS1* (Chapter 3) and *SCD1* (Chapter 4), which encode the enzymes polyketide synthase and scytalone dehydratase, respectively. Using the enzyme-deficient mutants, and a mutant deficient in the enzyme tetrahydroxynaphthalene reductase (4HNR), I investigate the role of melanin in the survival of the pathogen to environmental stresses (exposure to UV light, oxidative stress, extreme temperature and antifungal drugs) in Chapter 5. I then determine the sensitivities of the melanin-deficient mutants to killing by alveolar macrophages, and their pathogenicities in the invertebrate infection model *Galleria* (Chapter 6).

Chapter 2

2. General Materials and Methods

2.1 Fungal culture

The *Lomentospora prolificans* wild-type strain 3.1 (Thornton & Wills, 2015 b), gene disruption mutants and *Aspergillus fumigatus* strain AF293 were grown routinely on oatmeal agar (OA; O3506, Sigma, Sigma-Aldrich, Poole, Dorset, UK) or Sabouraud dextrose agar (SDA; Sabouraud dextrose broth (SBD; S3306, Sigma) containing 2% agar) at 30 ˚C under a 16 h fluorescent light regime to induce sporulation. Agar was sterilized by autoclaving at 121 ˚C for 15 min.

2.2 Nucleic acid analysis

2.2.1 Genomic DNA extraction

DNA extraction was carried out according to the procedures described in Alastruey-Izquierdo and co-workers (Alastruey-Izquierdo, *et al*., 2011). Spores were harvested by flooding OA plate cultures with 20 mL sterile autoclaved Milli-Q water (MQ-H_2O) and suspension with a sterile L-shaped spreader (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK). The suspension was filtered through sterile Miracloth (Calbiochem, San Diego, CA, USA) to remove hyphal tissue, centrifuged at 13,000 x g to pellet spores and the spores re-suspended in sterile MQ-H_2O. Sterile 75 cm2 tissue culture flasks containing 100 mL of tissue culture medium (TCM; RPMI-1640 medium (R0883, Sigma) containing 10% fetal bovine serum (Labtech International Ltd., Uckfield, East Sussex, UK), 2 mM L-glutamine (G7513, Sigma), penicillin and streptomycin) were inoculated with spore suspension to give a final concentration of 10^4 spores/mL and incubated with shaking (35 rpm) in an Innova 4000 rotary incubator (Eppendorf UK Ltd., Stevenage, Hertfordshire, UK) for 48 h at 30 ˚C.

Hyphal biomass was harvested by filtration through sterile Miracloth, washed with sterile MQ-H_2O, blotted dry with paper towel (Kimberley-Clark Ltd.,West Malling, Kent, UK) and stored at _80 ˚C until required. Frozen mycelium was ground to a fine powder in liquid N2 using a mortar and pestle, the powder transferred to 1.5 mL micro-centrifuge tubes and 800 µL of extraction buffer (0.2 M Tris–HCl, 0.5 M NaCl, 10 mM EDTA, SDS 1%) added followed by 800 µL of phenol:chloroform:isoamyl alcohol

mixture (25:24:1). The mixture was shaken gently for 30 s and then centrifuged at 14,000x g for 15 min at 4 ˚C. The upper layer was transferred to a fresh tube and mixed with an equal volume of phenol:chloroform:isoamyl alcohol mixture (25:24:1), vortexed for 30 s and centrifuged as described. The upper layer was combined with an equal volume of chloroform:isoamyl alcohol (24:1) and vortexed for 30 s. Following centrifugation for 5 min at 14,000x g, the upper layer was transferred to a fresh tube, the DNA precipitated by adding chilled isopropanol and pelleted by centrifugation at 4 ˚C for 15 min at 14,000x g. The pellet was washed with 70% ethanol and centrifuged for a further 10 min at 14,000x g. The pellet was dried for 15 min at 23 ˚C, re-suspended in 30 µL MQ-H_2O containing RNAse and then incubated for 1 h at 37 ˚C.

DNA quality and quantity was determined by both agarose gel electrophoresis and by using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.2 DNA manipulations

2.2.2.1 Digestion of plasmid and genomic DNA with restriction enzymes

Restriction enzymes were obtained from Promega UK Ltd. (Southampton, Hampshire, UK) or New England Biolabs (Ipswich, MA, USA). DNA (0.2–1.0 µg) was digested with 5 to 10 units of enzyme in a total volume of 30 µL. For Southern blot analysis, digestion was performed by incubating 50 µg of genomic DNA with 60 units of restriction enzyme in a total volume 50 µL. In both cases, the mixtures were incubated at 37 ˚C for 16 h.

2.2.2.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was used to amplify DNA fragments in an Applied Biosystems GeneAmp® PCR System 2400 cycler (Applied Biosystems, Foster City, CA, USA). GoTaq® Green Master Mix (Applied Biosystems, Foster City, CA, USA), Phusion High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) or Long PCR enzyme mix (Thermo Fisher Scientific, Waltham, MA, USA) were used according to the manufacturer's instructions.

For GoTaq, the reaction mixture contained 12.5 µL GoTaq Green Master Mix, 10 µM of forward and reverse primer, 50 ng of genomic DNA and nuclease free water to a total volume of 25 µL and PCR cycling conditions were an initial denaturation step at 98 ˚C for 3 min followed by 35 cycles at 98 ˚C for 1 min, 53.5–20 ˚C (depending on

annealing temperature of primers) for 1 min and at 72 ˚C, followed by a final extension step at 72 ˚C for 10 min.

Phusion High Fidelity DNA polymerase reaction mixture contained 5 µL of 5x Phusion HF buffer, 0.5 µL of 10 mM dNTPs, 0.25 µL Phusion, 10 µM of forward and reverse primer, 50 ng of genomic DNA and nuclease-free water to a final volume of 50 µL. PCR cycling conditions were an initial denaturation step at 98 ˚C for 30 s followed by 35 cycles of 98 ˚C for 10 s, 62 ˚C for 30 s and 72 ˚C for 45 s, with a final extension for 10 min at 72 ˚C. Long PCR enzyme mix was used for *PKS1* gene amplification. The PCR reaction mixture contained 5 µL of 10x Long PCR buffer with 15 mM $MgCl_2$, 1 µL of 10 mM dNTPs, 1 µL of 10µM of forward and reverse primer, 50 ng of genomic DNA, Long PCR enzyme mix to a total volume of 50 µL with nuclease-free water. PCR cycling conditions were an initial denaturation step at 94 ˚C for 3 min, 10 cycles at 94 ˚C for 20 s, 50 ˚C for 30 s and 68 ˚C for 7 min, followed by 25 cycles of 94 ˚C for 20 s, 50 ˚C for 30 s, 68 ˚C for 7 min. The final extension step was at 68 ˚C for 1 min.

2.2.2.3 Electrophoresis and purification of genomic DNA and PCR products

Agarose gel electrophoresis was used to fractionate digested DNA. The DNA was separated in 0.8% agarose gels and stained with ethidium bromide after mixing with loading dye (50 mL glycerol, 0.25 g bromophenol blue, 5 mL of 0.5 M EDTA (pH 8.0) and 45 mL MQ-H_2O). Electrophoresis was carried out in 1x Tris-borate EDTA buffer (TBE; 0.09 M Tris-borate, 0.002 M EDTA) for 1 h at 100 V. The DNA fragments were visualized using a UV transilluminator and fragment sizes was determined by comparison with a 1-kb ladder (Promega). Gel images were captured by using an Image Master VDS-CL gel documentation system (GE Healthcare Life Sciences, Little Chalfont, Buckinghampshire, UK) fitted with a Fujifilm FTI-500 Thermal Imaging system (GE Healthcare). DNA fragments were purified from agarose gels using Wizard® SV gel and PCR Clean-Up Systems (Promega) according to the manufacturer's instructions. Purified DNA was stored at –20 ˚C until required.

2.2.2.4 DNA cloning

2.2.2.4.1 DNA ligation

Ligation of DNA fragments was carried out using the In-Fusion® HD Cloning kit (Clontech Laboratories Inc., Mountain View, CA, USA) according to the manufacturer's instructions. The ligation reaction components were combined in a PCR tube in the following order: 2 µL 5x In-Fusion HD enzyme premix, 50 ng linearized vector, 200 ng purified PCR fragment and MQ-H_2O to a total volume of 10 µL. The cloning reaction was incubated at 50 ˚C for 15 min and then kept on ice until required.

2.2.2.4.2 Transformation of competent cells

Stellar competent Escherichia coli cells (Clontech) were thawed, placed on ice and mixed gently for even distribution. Fifty-µL of component cells were combined gently with 5 ng (2.5 µL) of In-Fusion cloning reaction. The tubes were incubated on ice for 30 min, heat-shocked at 42 ˚C for 45 s and then transferred to ice for 1–2 min. SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose) preheated to 37 ˚C was added to a total volume of 500 µL. The tube was incubated for 1 h at 37 ˚C with shaking (200 rpm) in an Innova 4000 rotary incubator. One hundred-µL were streaked on LB agar plates (LB broth (tryptone, 10 g/L, yeast extract, 5 g/L, NaCl, 10 g/L, pH 7.5) containing 18 g/L agar) overlaid with X-Gal (40 µg/mL) and supplemented with 1 mL of 100 µg/mL ampicillin. Plates were inverted and incubated at 37 ˚C for 16 h. Colonies were selected based on white-blue screening.

2.2.2.4.3 Plasmid isolation (Quick-Prep Method)

A single colony of transformed bacteria was used to inoculate 10 mL of LB broth containing 10 µL of 100 µg/mL ampicillin. The culture was incubated overnight at 37 ˚C with shaking (180 rpm) in an Innova 4000 rotary incubator (Eppendorf). A 1 mL aliquot of culture was transferred to a micro-centrifuge tube and, after centrifugation at 13,000x g for 1 min, the supernatant was discarded and the pellet re-suspended in 100 µL lysis solution (250 µL 1 M Tris-HCl (pH8.0), 200 µL 0.5 M EDTA, 170 µL, 60% sucrose, 2.4 mL MQ-H_2O) and vortexed. Two hundred-µL of alkaline solution (100 µL

10 M NaOH, 500 µL 10% SDS, 4.4 mL MQ-H_2O) was added and the samples were placed on ice for 10 min with occasional shaking. One hundred and fifty-µL of 3 M sodium acetate (pH 5.2) were added and the samples were incubated on ice for a further 10 min and then centrifuged (13,000x g) for 10 min at 4 ˚C. The supernatant was transferred to a fresh tube containing 1 mL of 100% ethanol and stored on ice for 10 min before centrifugation at 13,000x g for 10 min. This process was repeated with 70% ethanol, the pellet dried at 23 ˚C and then re-suspended in 30 µL of MQ-H_2O containing 7 µL RNase (10 mg/mL). The DNA was stored at –20 ˚C until used.

2.2.2.4.4 Plasmid DNA Isolation (Midi-Prep Method)

High quality plasmid DNA was isolated using the PureYield™ Plasmid Midiprep System (Promega) according to the manufacturer's instructions. A single colony of bacterial cells was grown in 50 mL LB containing 1 mL of a 100 µg/mL ampicillin solution for 16 h at 37 ˚C with shaking (180 rpm) in an Innova 4000 rotary incubator (Eppendorf). The cells were centrifuged at 5,000x g for 10 min and the supernatant discarded. The pellet was re-suspended in 3 mL of cell re-suspension solution
(50 mM Tris (pH 7.5), 10 mM EDTA, and 100 µg/mL of RNase). Three-mL of cell lysis solution (0.2 M NaOH, 1% (w/v) SDS) were added and the contents of the tube were mixed by inverting 3–2 times. After 3 min incubation at 23 ˚C, 5 mL of neutralization solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, pH 4.2) was added, the tube was inverted 5–20 times and the lysate centrifuged at 14,000x g for 15 min. The PureYield™ purification system was used to purify DNA according to the manufacturer's instructions and DNA stored at –20 ˚C until required.

2.3 Protoplast Transformation

Spores were harvested from 2-week-old OA cultures, inoculated into 100 mL TCM and incubated for 3 days at 30 ˚C with shaking (35 rpm) in an Innova 4000 rotary incubator. Hyphal biomass was harvested by filtration through Miracloth and washed and dried as described. The mycelium was transferred to a 50 mL Falcon tube containing 40 mL OM buffer/Glucanex (44 g $MgSO_4$_7H2O, 1.5 mL 10 mM $NaPO_4$, 1.8 g of 5% Glucanex (Novo Nordisk, Copenhagen, Denmark), pH 5.6) and incubated at 30 ˚C with shaking (75 rpm) for 3 h. The contents of the tube were transferred to sterile

polycarbonate Nalgene Oakridge tubes (Thermo Fisher) and the protoplasts were overlaid with chilled ST buffer (0.6 M Sorbitol and 0.1 M Tris-HCl (pH 7.0)). The protoplasts were centrifuged at 5000x g for 15 min at 4 ˚C using a swinging bucket rotor (Beckman JS-13.1, Beckman Coulter Inc., Brea, CA, USA) in a Beckman J2.MC centrifuge (Beckman Coulter). The protoplasts were recovered at the OM/ST interface and transferred to a sterile Oakridge tube, which was filled with cold STC buffer (1.2 M Sorbitol, 10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$). Protoplasts were pelleted at 3000x g for 10 min at 4 ˚C and washed twice with 10 mL STC, with complete re-suspension each time. Protoplasts were re-suspended in 1 mL STC and numbers quantified using a haemocytometer. Protoplasts were combined with 3 µg DNA in a sterile micro-centrifuge tube in a final volume of 150 µL and incubated for 15 min at 23 ˚C. One-mL of PTC (60% PEG4000, 10 mMTris-HCl (pH 7.5), 10 mM$CaCl_2$) was added and the contents were mixed by gentle inversion. The protoplasts were incubated at 23 ˚C for 15 min, added to 150 mL molten (45 ˚C) OCM agar (osmotically stabilized CM; 50 mL nitrate salt solution, 1 mL trace elements (22 mg/L zinc sulphate heptahydrate, 11 mg/L boric acid, 5 mg/L manganese(II) chloride tetrahydrate, 5 mg/L iron(II) sulphate heptahydrate, 1.7 mg/L cobalt(II) chloride hexahydrate, 1.6 mg/L copper(II) sulphate pentahydrate, 1.5 mg/L sodium molybdate dehydrate, 50 mg/L EDTA), 10 g glucose, 2 g peptone, 1 g yeast extract, 1 g casamino acids, 1 mL vitamin solution (0.001 g/L, biotin, 0.001 g/L, pyridoxine, 0.001 g/L, thiamine, 0.001 g/L riboflavin, 0.001 g/L, 0.001 g/L nicotinic acid), 273.84 g sucrose and 15 g agar in a final volume of 1 L, adjusted to pH 6.5 with 1 M NaOH) and the protoplast suspension poured into sterile 9 cm plastic culture dishes for incubation in the dark at 30 ˚C for 16 h. The plates were then overlaid with complete medium (CM; 10 g/L glucose, 2 g/L, peptone, 1 g/L, yeast extract (BD Biosciences UK, Oxford, Oxfordshire, UK), 1 g/L casamino acids, 0.1% trace elements, 0.1%, 0.1% vitamin solution, 6 g/L $NaNO_3$, 0.5 g/L, KCl, 0.5 g/L, $MgSO_4$, 1.5 g/L, KH2PO_4, (pH to 6.5 with NaOH), 15 g/L agar) containing 600 µg/mL hygromycin B (Calbiochem) and were then incubated for 1 week in the dark at 30 ˚C. Colonies resistant to hygromycin B were sub-cultured on CM containing 200 µg/mL hygromycin B and finally onto OA. For selection of sulfonylurea-resistant transformants, CM was replaced with BDCM medium (yeast nitrogen base without amino acids and ammonium sulfate, agar 1.7 g/L, ammonium nitrate, 2 g/L, asparagine, 1 g/L, glucose, 10 g/L, sucrose, 0.8 M, pH 6.0) with chlorimuron ethyl at

a concentration of 300 µg/mL in the overlay, chlorimuron ethyl at a concentration of 100 µg/mL in BDCM sub-cultures and finally growth on OA.

2.4 Southern Blotting

Southern blotting was carried out according to the protocol of Southern (Southern, 1975). Fifty-µg of genomic DNA was digested overnight using an appropriate restriction enzyme. The products of digestion were separated on 0.8% agarose gels at 100 V for 3 h. Gels were immersed in 0.25 M HCl for 15 min, followed by 0.4 M NaOH for a further 15 min, for de-purination and neutralization respectively. Gel blots were carried out by placing gels onto Whatman 3 mm paper wetted with 0.4 M NaOH and supported by a Perspex sheet with the ends of the paper immersed in 0.4 M NaOH. Gels were covered with Hybond-NX membrane (GE Healthcare Life Sciences), two layers of Whatman 3 mm paper and paper towel (Kimberley Clark). Finally, a 500 g weight was placed on the blotted gel and the blot was incubated for 16 h at 23 ˚C. Transferred DNA was UV-cross linked to the membrane using a BioLink BLX crosslinker (Sigma-Aldrich).

DNA probes were amplified by using Phusion HF buffer and the appropriate primers shown in Table 3.1 & 4.1 and reaction mixtures labelled with PCR DIG labelling mix (Sigma-Aldrich). Membranes were incubated in Hybaid hybridization bottles (Thermo Fisher) in a hybridization oven (Thermo Fisher) with Southern hybridization buffer (500 mL 1 M $NaPO_4$ (pH 7.0), 350 mL 20% SDS and Seradest added to a final volume of 1 L) at 62 ˚C for 30 min. Probes were denatured by boiling for 10 min and then added to hybridization bottles containing the membranes. Following hybridization at 62 ˚C for 16 h, the membranes were washed twice (15 min each) at 62 ˚C with Southern wash buffer (100 mL 1 M $NaPO_4$ (pH 7.0), 50 mL 20% SDS and MQ-H_2O to 1 L) and once at 23 ˚C using 20 mL DIG wash buffer 1 (6 mL Tween-20, 1.994 mL DIG buffer (maleic acid 0.1 M, NaCl 0.15 M, adjusted to pH 7.5 with 5 M NaOH) and with MQ-H_2O added to 2 L) for 5 min. Membranes were submerged in 50 mL of blocking buffer (1 g semi-skimmed milk powder and 100 mL DIG buffer 1) for 30 min at 23 ˚C and then incubated with 50 mL of antibody solution (2.5 µL anti-digoxigenin-AP Fab fragments (Sigma-Aldrich) and 50 mL blocking buffer) under the same conditions. After two 15 min washes with DIG wash buffer and equilibriation with 20 mL DIG buffer 3 (200 mL of 1 M Tris-HCl, 40 mL of 5 M NaCl, 100 mL of 1 M $MgCl_2$ (pH 9.5) with MQ-H_2O added to

2 L) for 5 min, membranes were incubated for 5 min with 2 mL CDP-star® chemiluminescent substrate solution (Sigma-Aldrich) and dried completely. Finally, the membrane was exposed to X-ray film (Fuji Photo Film (UK) Ltd., Manchester, Cheshire, UK) placed in a film cassette and incubated at 37 ˚C for 15 min. Films were developed using an OPTIMA X-Ray Film Processor (Protec GmbH, Oberstenfield, Germany).

2.5 Statistical Analysis

Unless otherwise stated, numerical data were analyzed using the statistical program Minitab (Minitab 16, Minitab®, Coventry, UK). Analysis of variance (ANOVA) was used to compare means of more than two data sets and Post-hoc Tukey-Kramer analysis was then performed to distinguish which sets were significantly different from one another. For comparisons of percentages, data was transformed using the arcsin_1 function prior to statistical analysis.

Chapter 3

Targeted deletion and complementation of the polyketide synthase-encoding gene *PKS1* in *Lomentospora prolificans*

3.1 Introduction

3.1.1 Fungal secondary metabolites

A characteristic feature of many filamentous fungi is their ability to produce secondary metabolites (Calvo, *et al*., 2002). These metabolites appear to have no direct role in growth, development and reproduction *per se*, and are especially generated during interactions of fungi with other organisms in the natural environment, perhaps contributing to their survival (Scharf, *et al*., 2014). The production, biological activities and manipulation of fungal secondary metabolites has received intense investigation ever since the discovery of the *Penicillium*-derived anti-bacterial compound penicillin by Alexander Fleming in 1929 (Fleming, 1929). Numerous secondary metabolites have been identified since that ground-breaking discovery, many of which have beneficial pharmaceutical properties such as antibiotics (Horgan, *et al*., 2011), while others such as aflatoxins and fumonisins that have toxic and carcinogenic effects (Li, *et al*., 2001). Recently, scientists have used novel genetic engineering approaches to improve beneficial secondary metabolite production (Meyer, 2008).

Determining the precise benefit of the secondary metabolites to the biology of the producing fungus remains a challenge (Yu, *et al*., 2005), with many correlative studies demonstrating a link between secondary metabolite production and morphological changes in fungi, especially during hyphal growth cessation and the induction of sporulation (Schimmel, *et al*., 1998). For example, deletion of *odeA*, a gene expressed during production of the fatty acid linoleic acid in *Aspergillus nidulans* (Calvo, *et al*., 2001), leads to a reduction in sporulation in the fungus but the precise reason for this is unclear.

The polyketide secondary metabolite tetrahydroxynaphthalene (1,3,6,8-THN), derived from acetyl coenzyme A (acetyl CoA) + malonyl coenzyme A (malonyl CoA), is a precursor in the biosynthesis of DHN-melanin (Figure 3.1), an essential component for fungal cell wall integrity (Pihet, *et al*., 2009). Soil-borne fungi are prone to ingestion

by predators, with melanised strains fungi such as *Cryptococcus neoformans* resistant to degradation the amoeba *Acanthamoeba* compared to non-melanised mutants (Steenbergen, *et al*., 2001). Molecular genetic studies have demonstrated the protective role of melanin and the secondary metabolite gliotoxin during predation of *Aspergillus fumigatus* by the amoeba *Dictyostelium discoideum* (Hillmann , *et al*., 2015). Furthermore, disruption of the gene *laeA*, which encodes the global regulator (LaeA) of secondary metabolite production in *Aspergillus nidulans* promotes fungivory of the fungus by the springtail *Folsomia candida* (Rohlfs, *et al*., 2007).

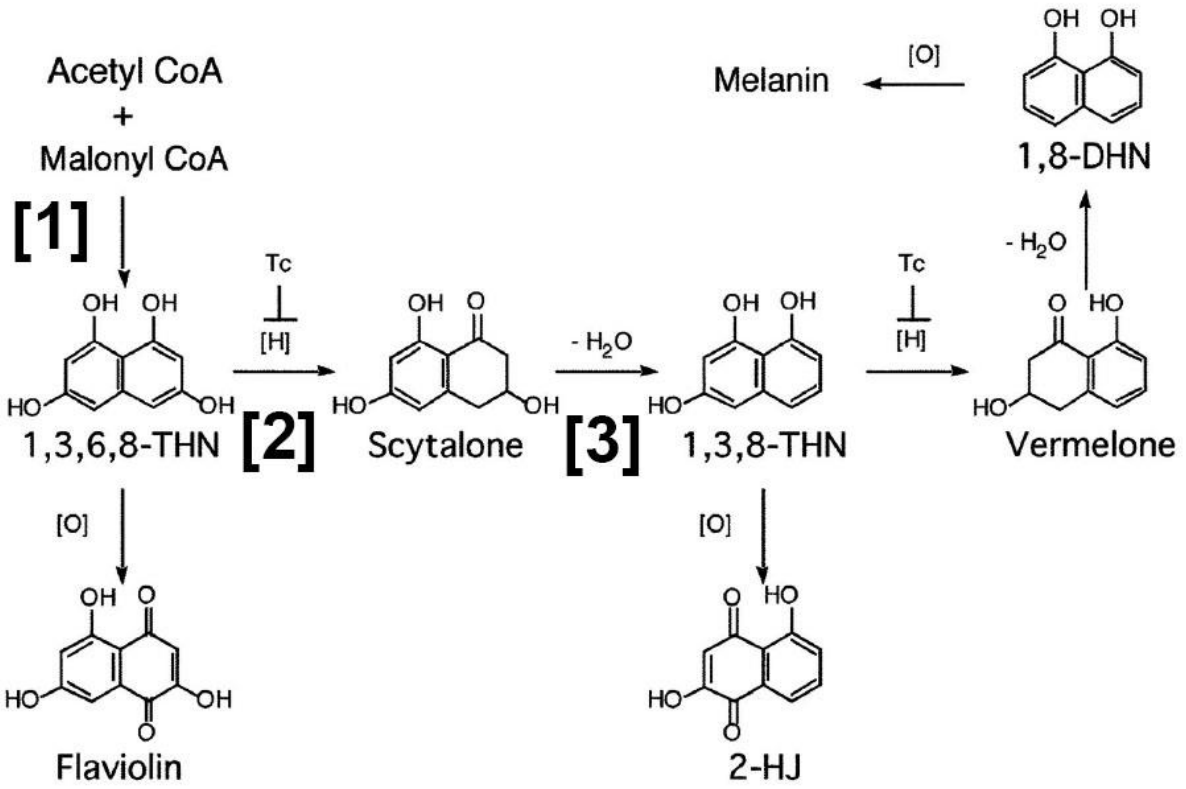

Figure 3.1. Schematic diagram showing the DHN melanin biosynthetic pathway in *L. prolificans*. Step (1) Acetyl CoA + Malonyl CoA to tetrahydroxynaphthalene (1,3,6,8-THN) is catalysed by the enzyme polyketide synthase (PKS). Mutants deficient in the enzymes tetrahydroxynaphthalene reductase and scytalone dehydratase, and which catalyse steps (2) and (3) respectively, are described in Chapter 4. Figure taken from Al-Laaeiby *et al*. (2016).

3.1.2 Polyketides and polyketide synthases

Keller and co-workers identify four categories of secondary metabolites based on their chemical structures (Keller, *et al*., 2005), with the genes responsible for their synthesis arranged within gene clusters (Keller, *et al*., 1997).

One of these categories is a large family of organic compounds produced by microorganisms known as polyketides (Tam, *et al*., 2015), which differ widely in their chemical structure and biological functions (Tam, *et al*., 2015). The polyketides include pigments (Brenner, *et al*., 2008), the statin drug Lovastatin (Alberts, *et al*., 1980), and antibiotics such as erythromycin, oxytetracycline and tetracenomycin (Stanzak, *et al*., 1986; Motamedi, *et al*., 1987; Binnie, *et al*., 1989). Other polyketides include toxic and carcinogenic compounds that contaminate food such as Aflatoxin B1 produced by *Aspergillus* spp. and which causes aflatoxicosis (Bennett, *et al*., 2003).

The name polyketide refers to the chemical nature of the organic compound, which is characterised by numerous keto groups alternately linked to carbon atoms (Hopwood, *et al*., 1990). Despite their varying structures, polyketides are derived via a common biosynthetic pathway (Schümann, *et al*., 2006) catalysed by polyketide synthase (PKS), an enzyme comprising, amongst others, ketosynthase (KS), acyl transferase (AT) and acyl carrier protein (ACP) domains (Fujii, *et al*., 2001; Schuemann, *et al*., 2009). After KS-catalysed condensation, another auxiliary domain participates in the further alteration of the β-keto group (Chan, et al., 2009) through ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) activities (Nair, *et al*., 2012).

Polyketide synthases are encoded by *PKS* genes which are located within gene clusters (Eisenman, *et al*., 2012); in eukaryotes this refers to a group of genes (and in bacteria an operon) located close to one another on a chromosome as members of a gene family (Winter, *et al*., 1999). Acetyl CoA is an essential starter unit in the biosynthetic process, with malonyl CoA participating as an extender unit in a process similar to fatty acid synthesis (a Claisen condensation) (Chan, *et al*., 2009).

Polyketide synthases are classified into three groups, PKS Type I, PKS Type II, and PKS Type III (Shen, 2003). The acyl carrier protein (ACP) domain has an indispensable role in Type I and Type II enzymes, whereas Type III enzymes use an ACP-independent mechanism (Shen, 2003). Type I PKSs are further sub-classified into iterative PKSs (re-use domains in a cyclic fashion) and modular PKSs (use a

sequence of separate modules and do not repeat domains) (Chan, *et al*., 2009). Iterative PKSs can be still further subdivided into NR-PKSs (non-reducing PKSs, the products of which are true polyketides), PR-PKSs (partially reducing PKSs), and FR-PKSs (fully reducing PKSs, the products of which are fatty acid derivatives) (Crawford, *et al*., 2010).

The advent of fungal molecular genetics and the widespread use of whole genome sequencing have revealed the wide numbers of PKS-encoding genes in different species. For example, there are twenty-five putative *PKS* genes in *Penicillium marneffei*, ten genes in *Coccidioides immitis*, and a single gene in the *Histoplasma capsulatum* (Collemare, *et al*., 2008; Woo, *et al*., 2010).

3.1.3 Polyketide synthases and melanin biosynthesis

In *Alternaria alternata*, there are three melanin biosynthesis genes located within a 30-kb gene cluster (Kimura & Tsuge, 1993), whereas six genes contribute to melanin biosynthesis in *Aspergillus fumigatus* and *Penicillium marneffei* (Kimura, *et al*., 1993 ; Tsai, *et al*., 1998; Woo, *et al*., 2010). The complexity of melanin biosynthesis therefore differs between different species of fungi (Brakhage, *et al*., 2005), but with close homology in enzyme-encoding gene sequences (Langfelder, *et al*., 2003).

Of the three types of PKS, Type I PKSs are involved in the synthesis of fungal DHN-melanin (Takano, *et al*., 1995). In *Wangiella dermatitidis*, the essential enzyme domains are β-ketoacyl synthase, acetyl-malonyl transferase, acyl carrier protein (ACP), and thioesterase (Feng, *et al*., 2001). PKS-encoding genes have been characterised in *Aspergillus fumigatus*, *Alternaria alternata and Wangiella dermatitidis*, with the genes *alb1*, *ALM1*, *WdPKS1* encoding Type I PKSs in these fungi, respectively (Takano, *et al*., 1997; Tsai, *et al*., 1998; Feng, *et al*., 2001). The length of *ALM1* gene in *Alternaria alternata* is almost 6-kb (Kheder, *et al*., 2012), while in *Aspergillus fumigatus,* the *alb1* gene is composed of five exons isolated by a short non-coding span (Langfelder, *et al*., 1998). Polyketide-deficient mutants of *Aspergillus fumigatus, Alternaria alternata, Penicillium marneffei* and *Colletotrichum lagenarium* display albino phenotypes and are sensitive to environmental stresses (Takano, *et al*., 1995; Langfelder, *et al*., 1998; Woo, *et al*., 2010; Kheder, *et al*., 2012).

3.1.4 Aims of Chapter 3

In this chapter, I set out to generate a mutant of *L. prolificans* deficient in the DHN-melanin biosynthetic enzyme PKS (Δ*Lppks1*::*hph*) by using a split-marker technique of homologous recombination, and re-introduction of the *PKS1* gene into the Δ*Lppks1*::*hph* mutant to generate a complemented strain Δ*Lppks1*::*hph*:PKS. I then compare the phenotypes (growth habit, sporulation and pigmentation) of the two mutants to the wild type strain 3.1. I investigate the consequences of *PKS1* gene deletion and complementation (and loss and recovery of melanisation in the Δ*Lppks1*::*hph* and Δ*Lppks1*::*hph*:PKS mutants respectively) to pathogen survival in Chapters 5 and 6, alongside mutants described in Chapter 4 that are deficient in the melanin biosynthetic enzymes scytalone dehydratase and tetrahydroxynaphthalene reductase.

3.2 Material and Methods

3.2.1 Multiple DNA sequence alignments

The DNA sequence of the polyketide synthase-encoding gene *PKS1* was obtained by interrogation of the *L. prolificans* strain 3.1 (Thornton, *et al*., 2015b) full genome sequence archived at Biosciences, University of Exeter. The program Basic Local Alignment Search Tool (BLAST) was used to explore the sequence homologies between the ORF of the *PKS1* gene of *L. prolificans* and *PKS1* gene sequences from other species deposited in public database (*Pseudallescheria boydii, Colletotrichum higginsianum*, *Scedosporium apiospermum*, *Monascus purpureus,*). Sequence alignments were carried out by using DNAMAN software (http://www.lynnon.com/dnaman.html). This software identifies identical, similar and non-identical nucleotides.

3.2.2 Primer design

The full genome sequence of *L. prolificans* strain 3.1 archived at Biosciences, University of Exeter was used to design primers for disruption and complementation of the gene *PKS1*, and are shown in Table 3.1. Primers were constructed by using the online resource (http://depts.washington.edu/bakerpg/primertemp/primermelttemp.html) and for reverse complements (http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html).

Table 3.1. Details of primer sequences used in the disruption and complementation of the *L. prolificans* PKS-encoding gene *PKS1*.

Primer Name	Sequence 5'-3'	Product
Lppks1-F	TCTCGGTTTCTCCATGCAAA	*Lppks1* ORF
Lppks1-R	CCTGCACAAACAATTCGTTA	*Lppks1* ORF
Lppks1-LFF	AATCAAGTCGCCAGGACCTT	*Lppks1* LF
Lppks1-LFR	GTCGTGACTGGGAAAACCCTGGCGTTTG CATGGAGAAACCGAG	*Lppks1* RF
Lppks1-RFF	TCCTGTGTGAAATTGTTATCCGCTGAGC TGTATCACTGAGACA	*Lppks1* RF
Lppks1-RFR	GAGGCCATTCAAAGATCCCA	*Lppks1* RF
HY split	GGATGCCTCCGCTCGAAGTA	*Lppks1* LF + HY
YG split	CGTTGCAAGACCTGCCTGAA	*Lppks1* YG + RF
M13F	CGCCAGGGTTTTCCCAGTCACGAC	-
M13R	AGCGGATAACAATTTCACACAGGA	-
Lppks1-f1	TAGAACTAGTGGATCCGTCTCACATCGT CGTACTATAAT	*Lppks1* gene fragment
Lppks1-r1	CGGTATCGATAAGCTCGGCACTAGGATA TAAACCCTCTT	*Lppks1* gene fragment
Lppks1-ComF	GAGTTCTGCCGTCAGGGAAA	*Lppks1* probe
Lppks1-ComR	GAAGATGTGGCGCATGGTAG	*Lppks1* probe

The reverse complement of M13 forward and reverse sequences, used with HY split and YG split respectively, are shown underlined. ORF: open reading frame; LF: left flank; RF: right flank; LFF: left flank forward; LFR: left flank reverse; RFF: right flank forward; RFR: right flank reverse.

3.2.3 Targeted disruption of the *PKS1* gene and generation of Δ*Lppks1*::*hph* mutant

Targeted gene disruption was carried out following the split marker method (Yu *et al*., 2004) using the protoplast transformation procedure outlined in Section 2.3. For targeted replacement of the PKS-encoding gene *PKS1*, two rounds of PCR were required to complete the process (Figure 3.2) In the first round PCR, the left (upstream)(1.920-kb) and right (downstream)(1.913-kb) flanking regions of the *PKS1* ORF were amplified from *L. prolificans* genomic DNA (Section 2.2.1) using primer pairs Lppks1-LFF/Lppks1-LFR and Lppks1-RFF/Lppks1-RFR designed to include an extension complementary to the hygromycin phosphotransferase gene (*HPH*) conferring resistance to the antibiotic hygromycin B (Table 3.1). The left and right fragments of the *HPH* gene were amplified from pCB1004 with primer pairs HY split/M13F and M13R/YG split, respectively. In a second round PCR, the left flank was fused with one half of the hygromycin cassette (HY)(1.184-kb) and the right flank with the other half (YG)(0.773-kb). The second round PCR products LF+HY (3.104-kb) and RF+YG (2.686-kb) were used for protoplast transformation (Section 2.3). Homologous recombination resulted in the replacement of the *PKS1* ORF with the functional *HPH* gene. Putative Δ*Lppks1*::*hph* transformants were selected based on resistance to 200 µg/ml of the antibiotic hygromycin B (Section 2.3).

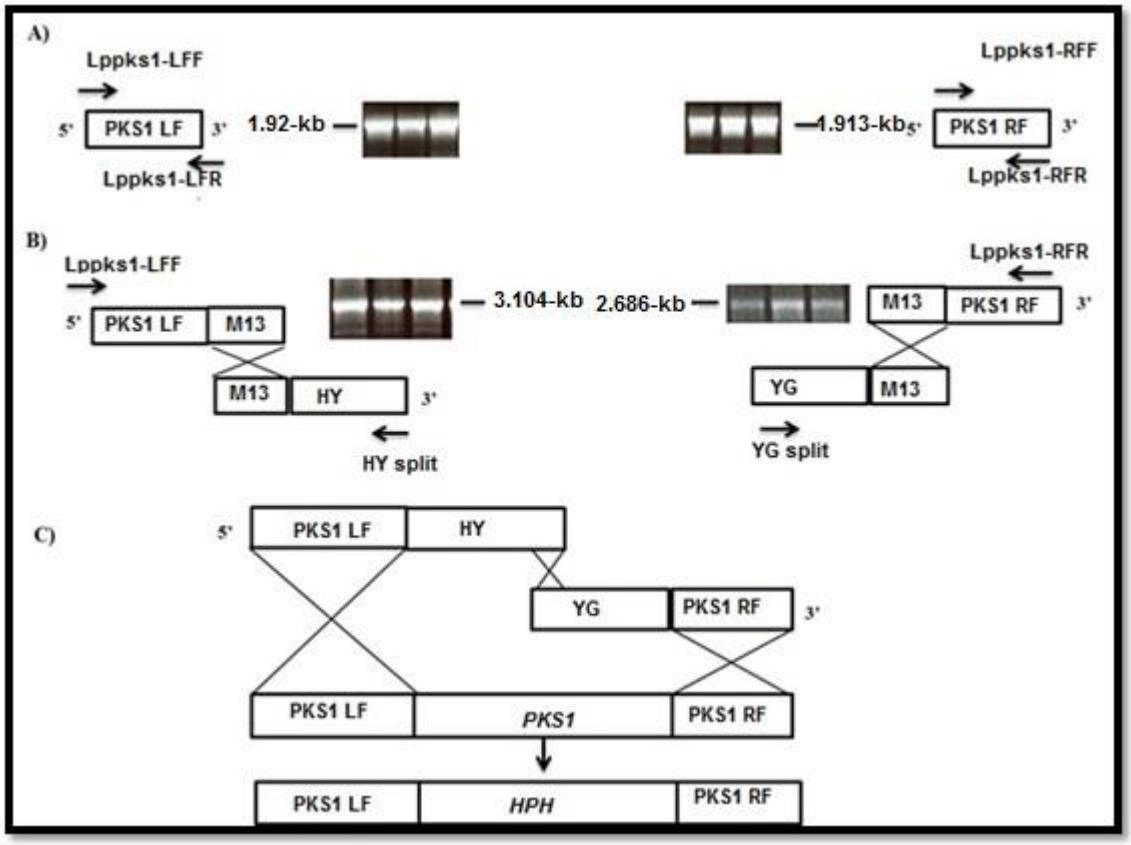

Figure 3.2. Schematic representation of the targeted replacement strategy for the PKS-encoding gene *PKS1*. A) First round PCR: the left (upstream)(1.920-kb) and right (downstream)(1.913-kb) flanking regions of the *PKS1* ORF were amplified using the primers shown. **B)** Second round PCR was carried out to fuse the flanks of *PKS1* with overlapping fragments of the hygromycin cassette (HY and YG). **C)** Homologous recombination event in which the HPH cassette replaces the *PKS1* ORF and is integrated into the sequence, thereby disrupting the PKS-encoding gene.

3.2.4 Southern blotting

Hygromycin B resistant mutants were investigated further by Southern blot analysis (Section 2.4). Genomic DNA from putative Δ*Lppks1*::*hph* mutants and the wild-type strain 3.1 were digested with the restriction enzyme *Hpa*I according to the methods outlined in Section 2.2.2.1. The products were separated by agarose gel electrophoresis and blotted onto a Hybond-NX membrane (Section 2.4). The membrane was probed with a 1.913-bp right flank fragment of the *PKS1* ORF. Putative Δ*Lppks1*::*hph* mutants were identified by a fragment size of 5.6-kb compared to the 6.5-kb fragment of the wild-type strain. For Southern blot analysis of putative Δ*Lppks1*::*hph*:PKS complementation mutants, genomic DNA was digested with the restriction enzyme *Bgl*II, fractionated by gel electrophoresis and blotted onto Hybond-NX membrane. The membrane was probed with a 0.8-kb fragment of the *PKS1* ORF generated using the primers Lppks1-ComF and Lppks1-ComR (Table 3.1).

3.2.5 Complementation of the Δ*Lppks1*::*hph* mutant

An albino Δ*Lppks1*::*hph* mutant was complemented by integration of a DNA fragment consisting of 2.992-kb of promoter region, 6.708-kb of the ORF and 0.603-kb terminator region of the *PKS1* gene to restore gene functionality and concomitant melanin biosynthesis. A schematic diagram illustrating the complementation procedure is shown in Figure 3.3. The *PKS1* fragment was amplified by PCR using the gene-specific primers Lppks1-f1 and Lp-pks1-r1 (Table 3.1), designed to include 15-bp of sequence homologous to the ends of the linearized vector pCB1532. The vector pCB1532, which contains the sulfonylurea resistant allele of the *Magnaporthe oryzae ILV1* gene (Sweigard, et al., 1997), was linearized digestion by with the restriction enzymes *Bam*HI and *Hin*dIII. The PCR product was ligated to linearised pCB1532 vector and used to transform competent *E. coli* cells (Section 2.2.2.4.2), which were selected based on white-blue screening (Section 2.2.2.4.2). Plasmid DNA was purified (Section 2.2.2.4.4) and sequenced to confirm the correct *PKS1* insertion and the vector then transformed into the Δ*Lppks1*::*hph* mutant. Putative Δ*Lppks1*::*hph*:PKS complementation mutants were selected based on resistance to 100 µg/ml sulfonylurea.

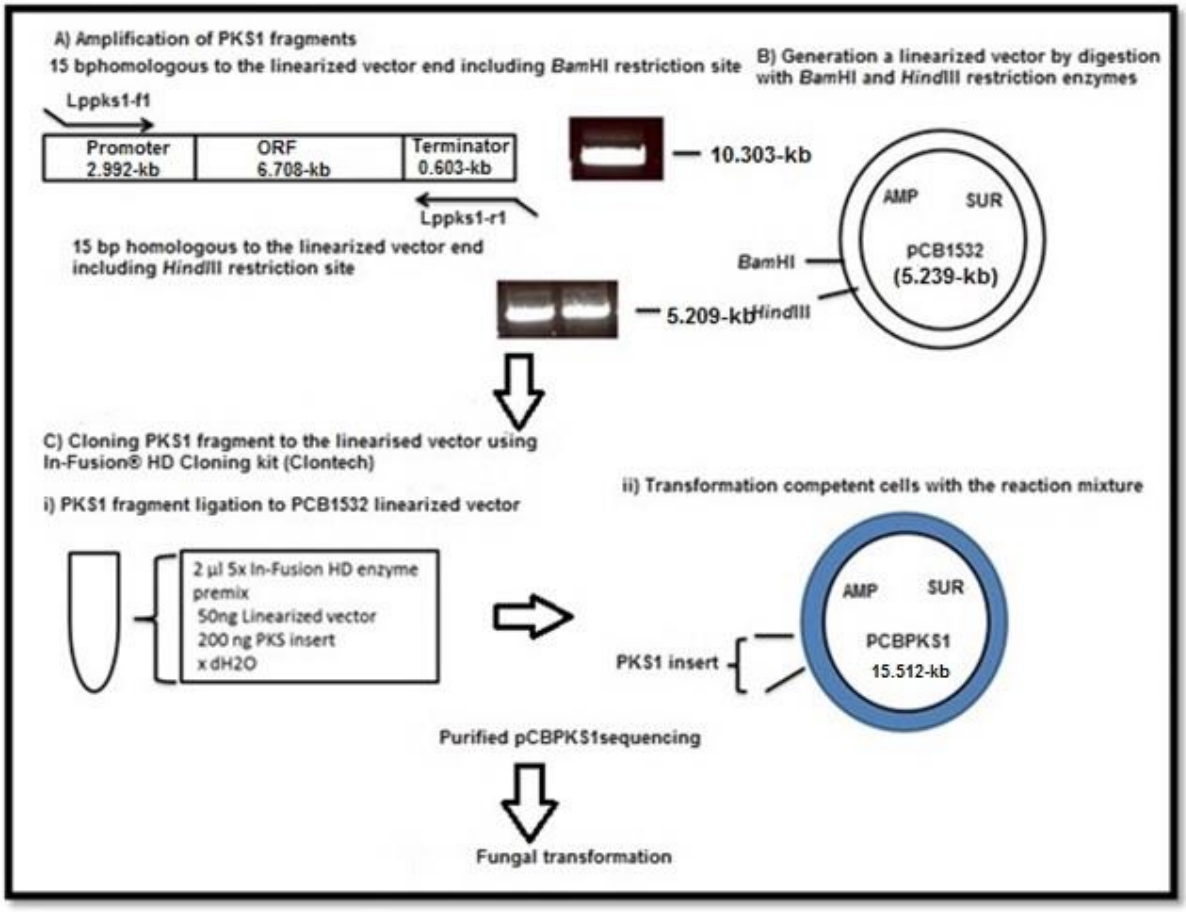

Figure 3.3 Schematic diagram showing procedure for complementation of the Δ*Lppks1*::*hph* mutant. A) Amplification of the 10.303-kb fragment of the *PKS1* gene consisting of promoter region (2.992-kb), ORF (6.708-kb) and terminator (0. 602-kb) using primers designed to include 15-bp of sequence homologous to the ends of the linearised vector pCB1532. **B)** Digestion of pCB1532 *Hin*dIII and *Bam*HI restriction enzymes to create the linearised vector. **C)** Cloning the *PKS1* fragment to the linearised pCB1532 vector; the *PKS1* fragment is ligated to the vector and the reaction mixture used to transform Stellar competent cells. Once confirmed by sequencing, the vector is used to transform fungal protoplasts for generation of complemented mutants.

3.2.6 Hyphal growth, sporulation and pigmentation

Stable transformants were generated following three rounds of single spore isolation under antibiotic selection (hygromycin or sulfonylurea). Spores were harvested as described (Section 2.2.1) and spore suspensions serially diluted before spreading on CM/BDCM selection plates and incubation for 3 days at 30 ^{o}C in the dark. Colonies developing from single spores were sub-cultured and the process repeated twice more. For hyphal growth determinations, strains were inoculated centrally onto replicate OA plates and colony diameters were measured over a 2-wk growth period at 30 ^{o}C, with spore production quantified after 14 d. Spores were suspended in 20 ml dH_2O using plastic L-shaped spreaders, filtered through Miracloth, and spore concentrations determined using a haemocytometer. There were 3 replicates for each experiment and experiments were repeated 3 times.

3.3 Results

3.3.1 *PKS1* gene sequence and homology to other fungal *PKS1* genes

The nucleotide sequence of the *PKS1* gene recovered from the *L. prolificans* 3.1 whole genome sequence is shown in Figure 3.4. The ORF of the *L. prolificans PKS1* gene is 6,708-bp long and is interrupted by two introns (Figure 3.4). Intron I spans the region 293-391 bp (highlighted in yellow), and Intron II spans the region 684-743 bp (highlighted in blue), from the start of the ORF. The lengths of Intron I and II are 98-bp and 59-bp, respectively. A sequencing error in the ORF (indicating by the sequence of Ns highlighted in red) meant that an accession number for the *PKS1* gene could not be obtained from NCBI.

GGCGAAGTTAATCCTCTGCAGATTGAGATAGGAGATTGGGATGTCTAGTGACGAAGACTG
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AGGATCTCGGACGTCGTTGCGGCGCTCAGATAATTGCTTTGGCAATCCACAGTGATTAGA
CCGAGCTGATGACACGATGCAAACCGTATAACCCCTCGAACCGGACTTGTATGCATGGCC
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CGTCCCATCGCCAGCTGGCTCTTCTTGCCAGTCTAAAATTCATTTCATCCTTCATATGTC

Figure 3.4. Nucleotide sequence of the *L. prolificans* polyketide synthase-encoding gene *PKS1* with flanking regions. The open reading frame of the gene is shown in bold type and highlighted in grey, and the start (ATG) and stop (TGA) codons are underlined. Intron I and Intron II are highlighted in yellow and blue, respectively. The Ns highlighted in red indicate a sequence error.

The translated PKS1 protein is 2169 amino acids in length (Figure 3.5) and has eight domains (multi-domain protein) including starter unit, (1) acyl-carrier protein ACP transacylase (SAT) (8-245 aa), (2) ketoacyl-synt (382-631aa), (3) ketoacyl-synt-C (639-760 aa), (4) acyl transferase domain (912-1212 aa), (5) polyketide synthase dehydratase (1320-1604 aa), (6) phosphopantetheine attachment site (1661-1727 aa), (7) PP-binding (1788-1854 aa), and (8) thioesterase (1929-2165 aa). The protein sequence shows that the PKS1 protein in *L. prolificans* is Type I PKS1.

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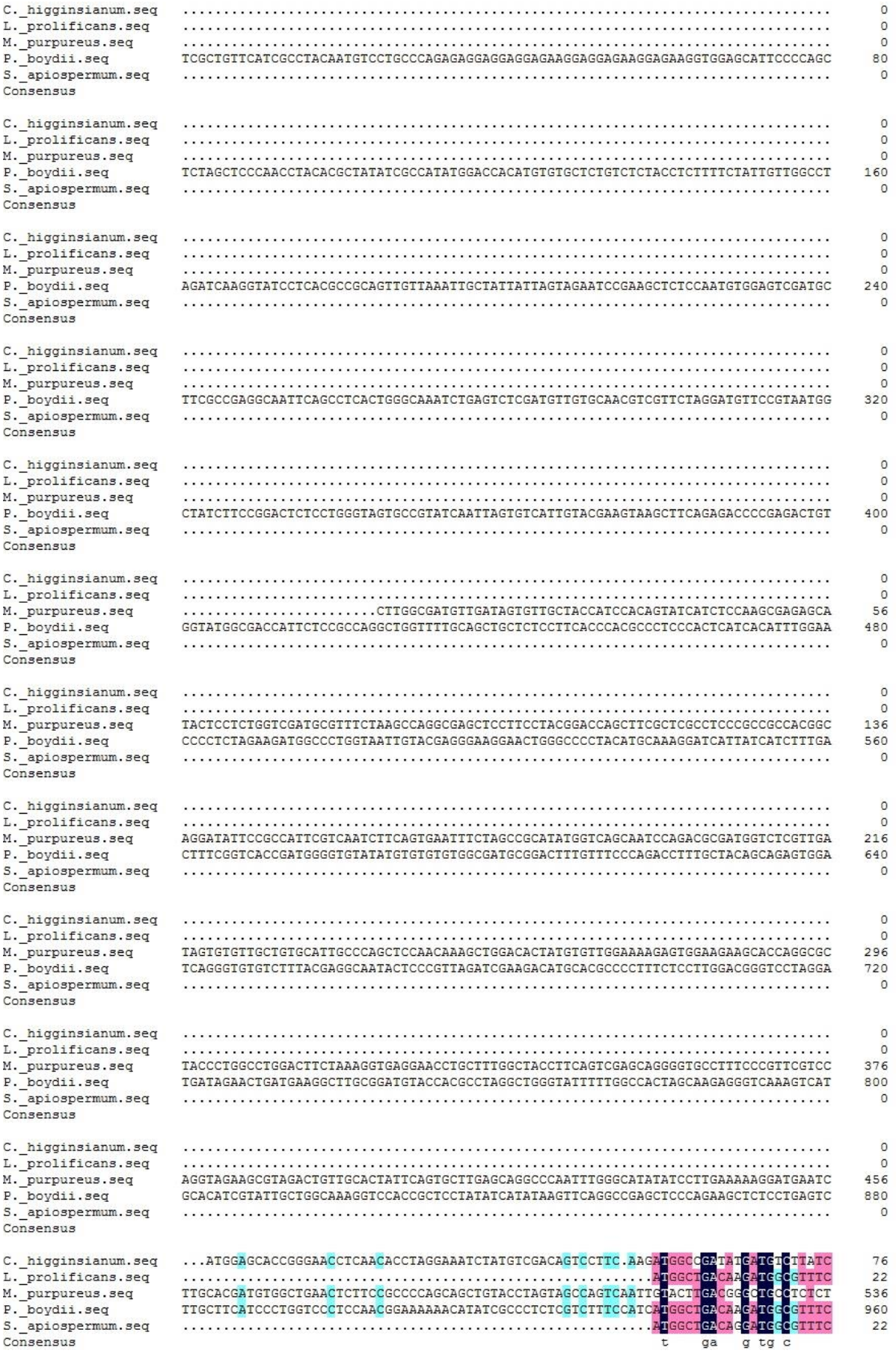
KLPTDPRPDPYPTGHALFLLDNRTDFGPNRWDDYLDYKRIRTMHMPGNHFSMMHGDLVSV
HHLTSFFSF

The program Basic Local Alignment Search Tool (BLAST) was used to investigate homology of the *PKS1* gene with comparable gene sequences deposited in public database. The ORF of the *L. prolificans PKS1* gene (Figure 3.4) when aligned against other ORFs (Figure 3.6), was shown to have 78% homology with *Pseudallescheria boydii*, 72% with *Colletotrichum higginsianum*, 74% with *Monascus purpureus*, 82% with *Scedosporium apiospermum*, 79%. Using DNAMAN software, identical nucleotides are shown in blue, similar nucleotides are shown in pink, while non-identical nucleotides are unshaded.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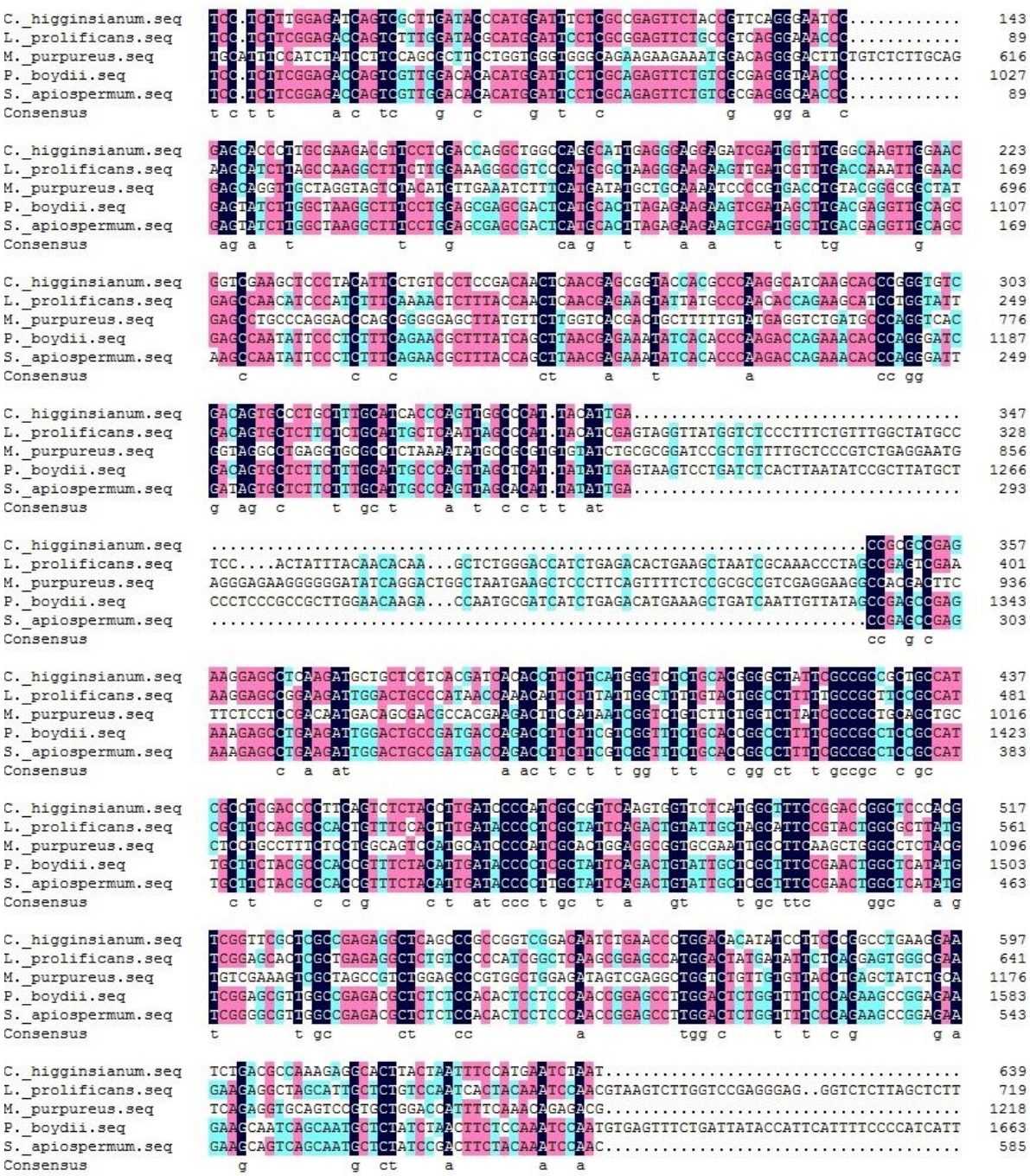
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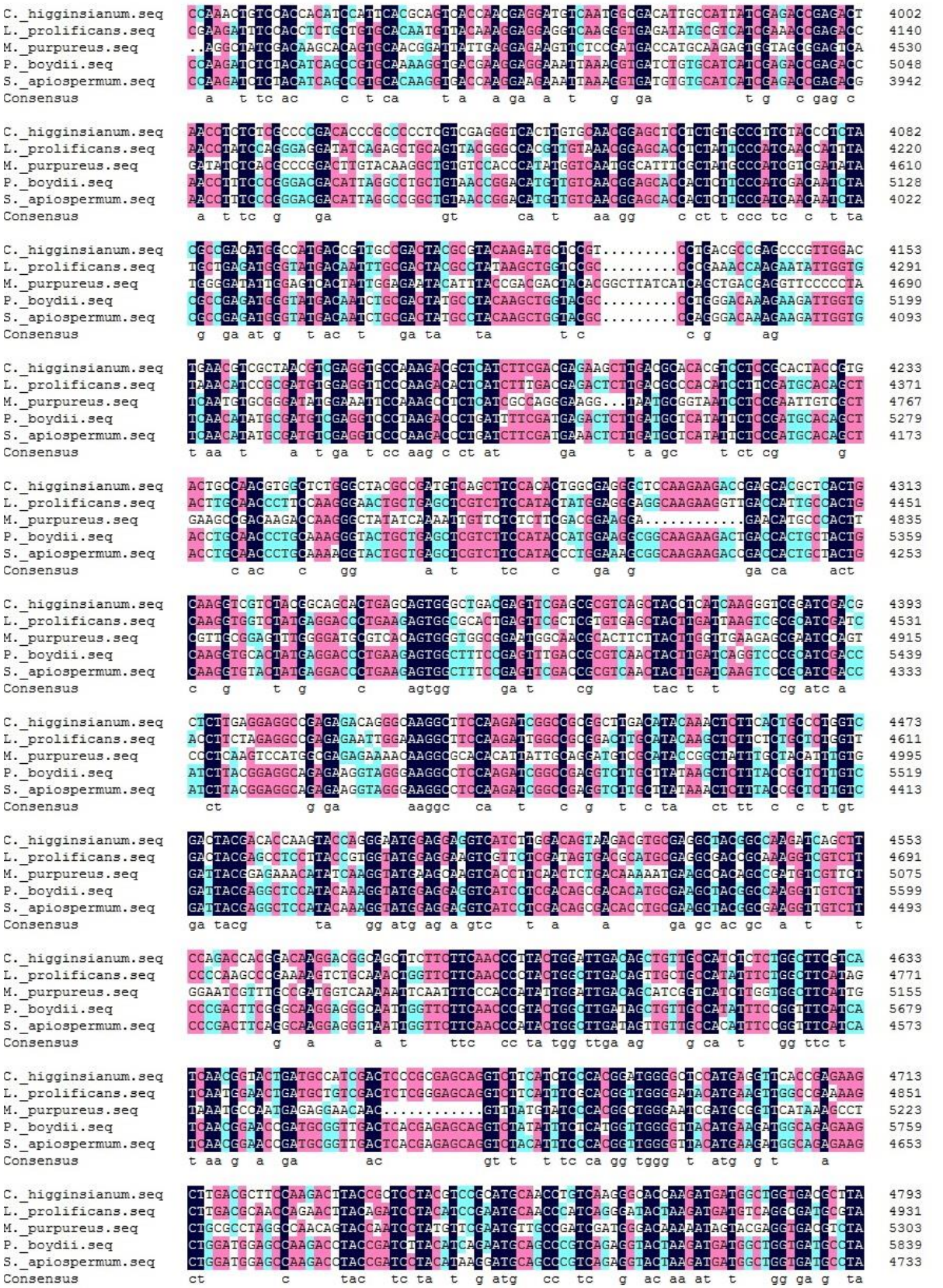

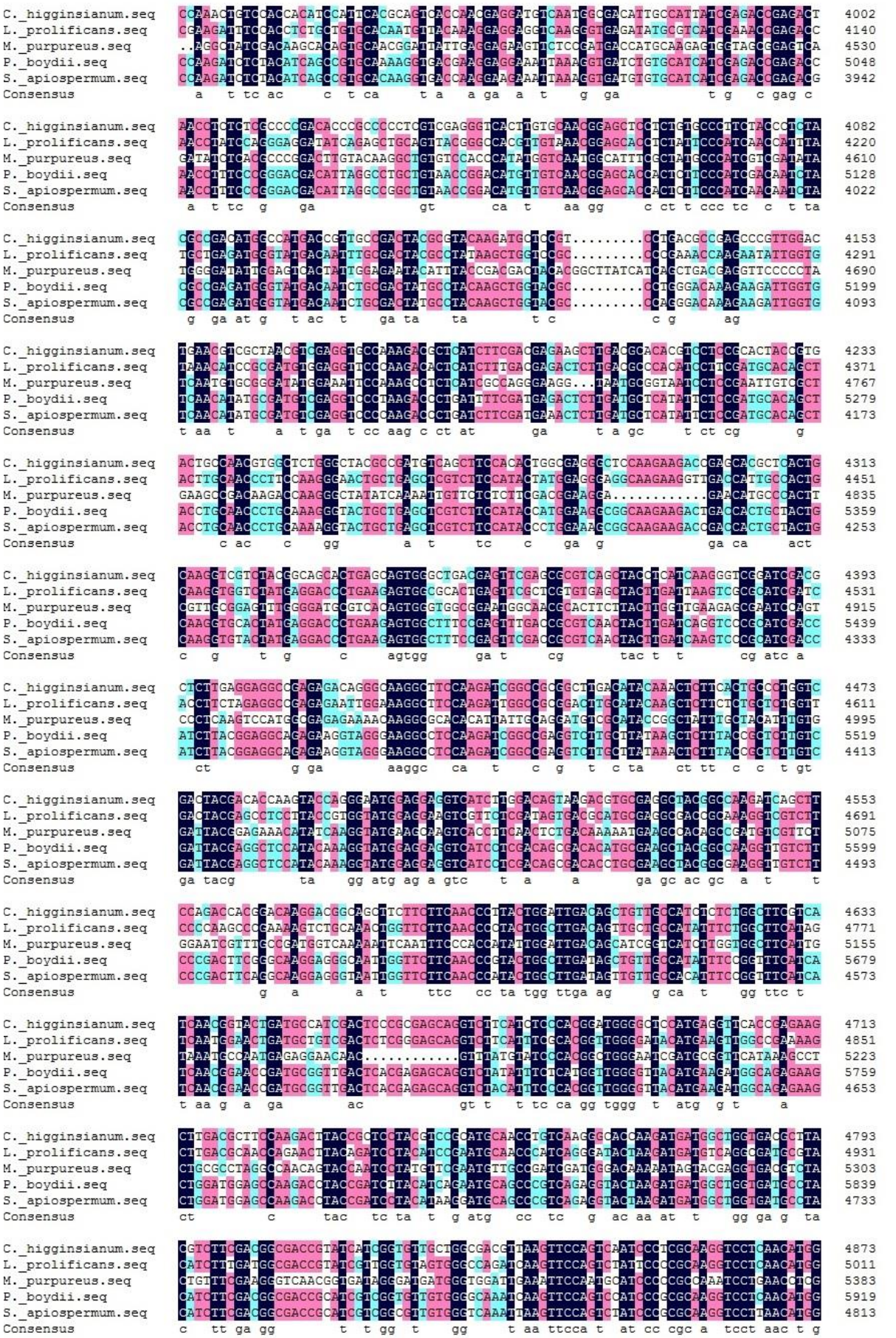
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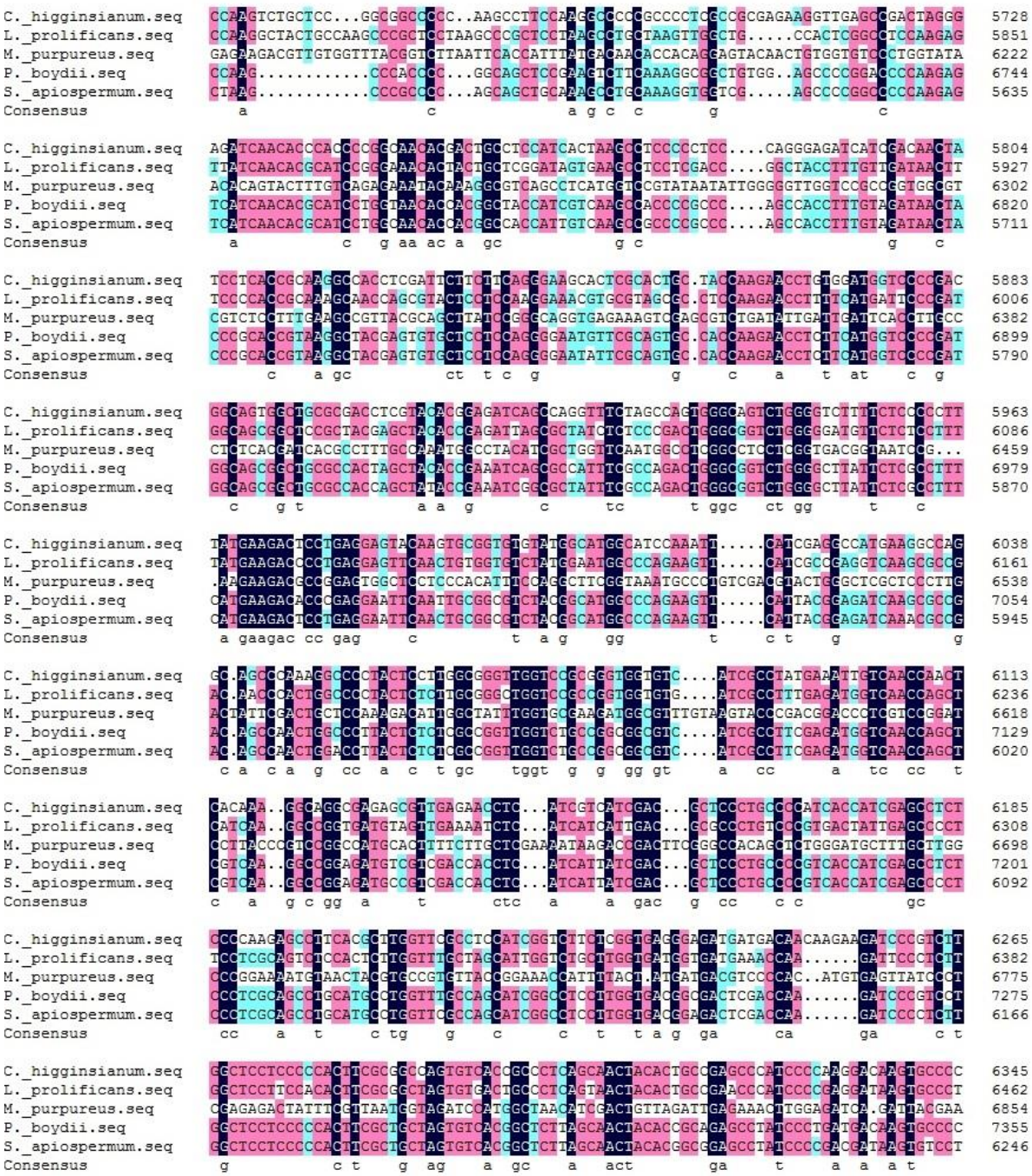
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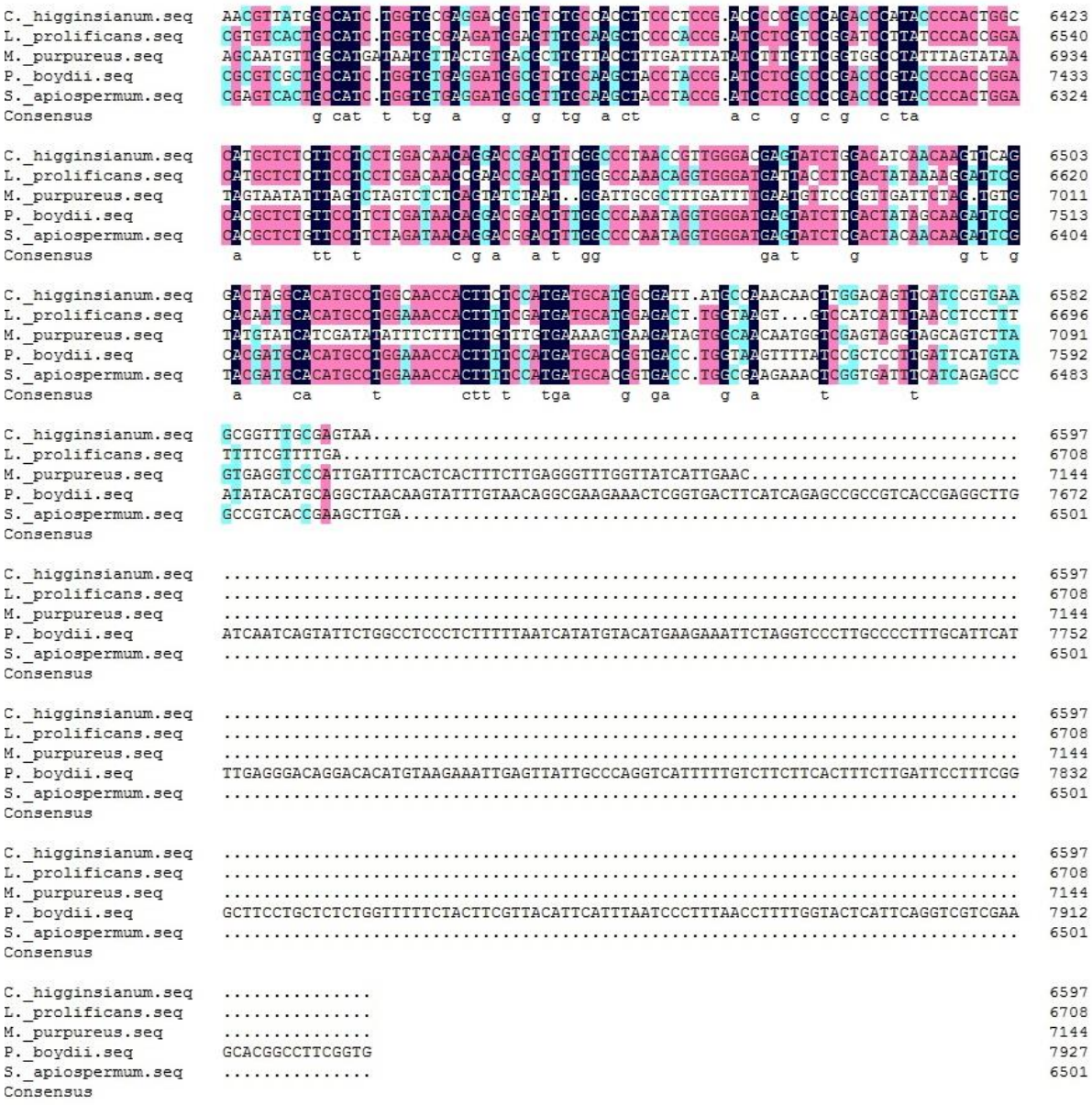

Figure 3.6. Nucleotide sequence alignment of the *L. prolificans PKS1* gene against comparable PKS-encoding gene sequences from *Colletotrichum higginsianum*, *Monascus purpureus, Pseudallescheria boydii*, and *Scedosporium apiospermum*. Identical nucleotides are shown in blue, similar nucleotides in pink and non-identical sequences are un-shaded.

3.3.2 Generation of Δ*Lppks1*::*hph* mutant

Putative transformants were identified following re-generation of transformed protoplasts in the presence of 200 µg/ml of the antibiotic hygromycin B. Figure 3.7 shows growth of a putative Δ*Lppks1*::*hph* mutant following protoplast re-generation and sub-culture onto complete medium containing 200 µg/ml hygromycin.

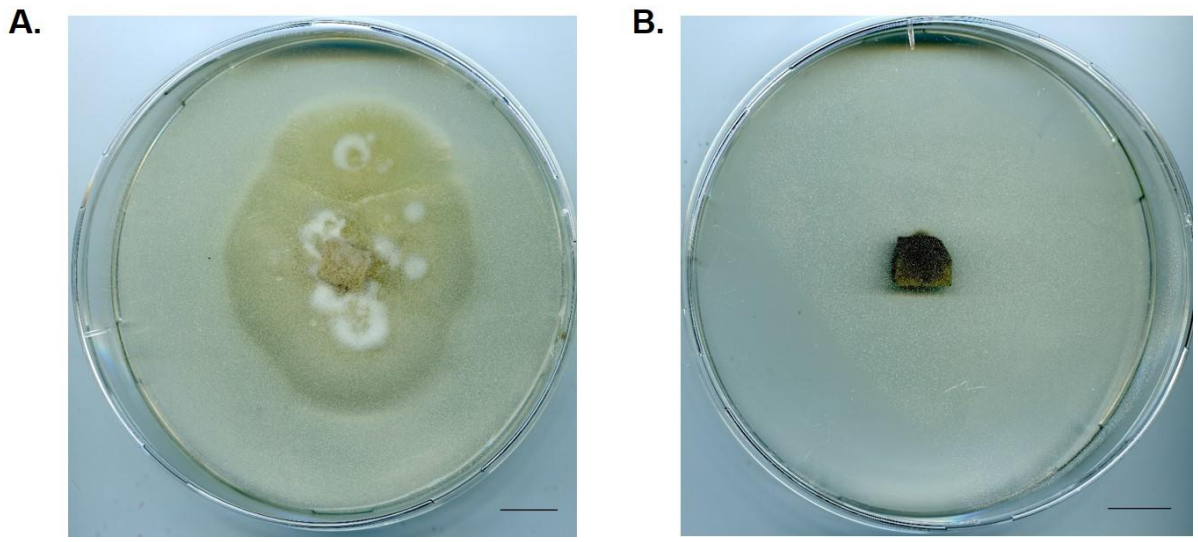

Figure 3.7 Growth of the wild type *L. prolificans* strain 3.1 and a putative Δ*Lppks1*::*hph* transformant on complete medium (CM) supplemented with 200 µg/ml hygromycin at 30 ºC for 7 days. Note growth of the putative Δ*Lppks1*::*hph* mutant **(A)**, but lack of growth of the wild-type strain **(B)**. Wild type strain 3.1 sensitive to hygromycin. Image was captured using an Epson Expression 1680 Pro scanner. Scale bars = 1 cm.

3.3.3 Southern blotting analysis

Genomic DNA of the wild-type strain 3.1 and putative mutants was digested with the restriction enzyme *Hpa*I (Figure 3.8A) and probed with a 1.913-kb right flank fragment of the *PKS1* ORF (Figure 3.8B). Putative Δ*Lppks1*::*hph* mutants were identified by a fragment size of 5.6-kb compared to the 6.5-kb fragment of the wild-type strain. Accordingly, two Δ*Lppks1*::*hph* mutants (Figure 3.8B, lanes 5 and 7 indicated by white asterisks) were identified with the correct size of 5.6-kb, compared to the 6.5-kb of the wild-type strain (Figure 3.8B, lane 1 indicated by the black asterisk).

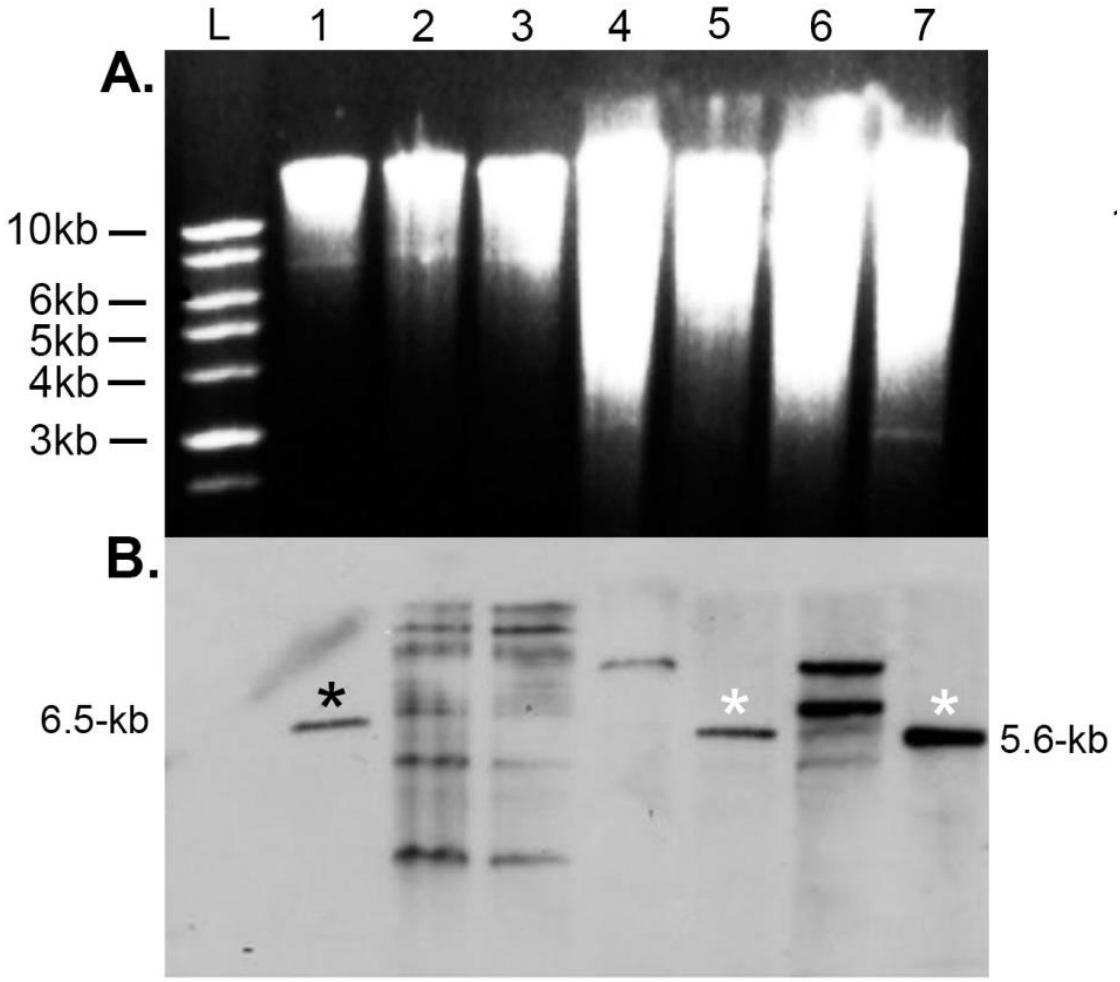

Figure 3.8. Southern blot analysis of the targeted gene replacement of *PKS1*. (A) Genomic DNA of the *L. prolificans* wild-type strain 3.1 (lane 1) and putative Δ*Lppks1*::*hph* transformants (lanes 2 to 7) were digested with the restriction enzyme *Hpa*I, fractionated by gel electrophoresis and blotted onto Hybond-NX membrane. **(B)**. Lane L contains DNA size marker. The membrane was probed with a 1.913-kb right flank fragment of the *PKS1* ORF. The presence of single 5.6-kb bands in lanes 5 and 7, compared to the single 6.5-kb band in lane 1, indicates successful replacement of the *PKS1* gene in these two strains.

3.3.4 Complementation of the Δ*Lppks1*::*hph* mutant and Southern blotting analysis

The Δ*Lppks1*::*hph* mutant corresponding to lane 7 in Figure 3.8 was complemented by random integration of a 10.303-kb *PKS1* fragment into the mutant genome. The fragment was amplified from the wild-type strain 3.1 using primers Lppks1-f1 and Lp-pks1-r1 (Table 3.1). By using the IN-Fusion® HD Cloning Kit (Clontech), the *PKS1* fragment was cloned into pCB1532 linearized vector (Section 2.2.2.4). The cloned vector was purified (Section 2.2.2.4.4) and sequenced to confirm correct *PKS1* insertion into the pCBPKS1 vector (Figure 3.9). The vector was then transformed into the Δ*Lppks1*::*hph* mutant corresponding to lane 7 of Figure 3.8, and transformants were selected based on resistance to 100 µg/ml sulfonylurea. Using this procedure, a single putative complemented strain (∆*Lppks1::hph:*PKS) was recovered (Figure 3.10). Genomic DNA of wild-type 3.1, Δ*Lppks1*::*hph* and the putative complemented mutant ∆*Lppks1::hph:*PKS was digested with the restriction enzyme *Bgl*II, fractionated by gel electrophoresis (Figure 3.11A) and blotted onto a Hybond-NX membrane (Figure 3.11B). The membrane was probed with a 0.8-kb fragment of the *PKS1* ORF. The probe hybridised to a 7.3-kb fragment present in the wild-type strain 3.1 and complemented strain (Figure 3.11B, lanes 1 and 4 respectively), which was absent in the two Δ*Lppks1*::*hph* mutants (Figure 3.11B, lanes 2 and 3). The presence of a single 7.3-kb band in strain Δ*Lppks1*::*hph*:PKS (comparable in size to strain 3.1) indicates successful complementation of the Δ*Lppks1*::*hph* mutant.

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C";

Figure 3.9. Nucleotide sequences of the *PKS1* fragments cloned into the pCB1532 vector for *PKS1* gene complementation of the Δ*Lppks1*::*hph* mutant. The pCBPKS1 vector was purified and sequenced to confirm successful cloning of the *PKS1* fragment (10.303-kb). The sequencing was performed to identify the three *PKS1* fragments.

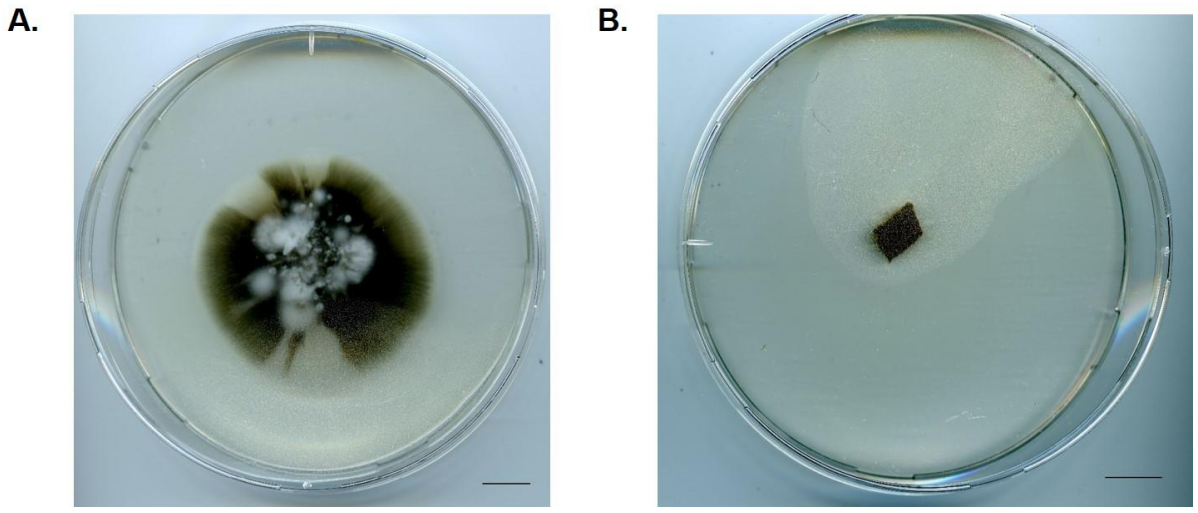

Figure 3.10. Identification of ∆*Lppks1::hph:PKS* complement mutants using sulfonyl urea. (A) Growth of a putative antibiotic-resistant ∆*Lppks1::hph:*PKS strain on BDCM medium supplemented with 100 µg/ml sulfonyl urea. **(B)** Lack of growth of Δ*Lppks1*::*hph* showing the non-complemented mutant is sensitive to the antibiotic. Images were captured using an Epson Expression 1680 Pro scanner. Scale bars = 1.5 cm.

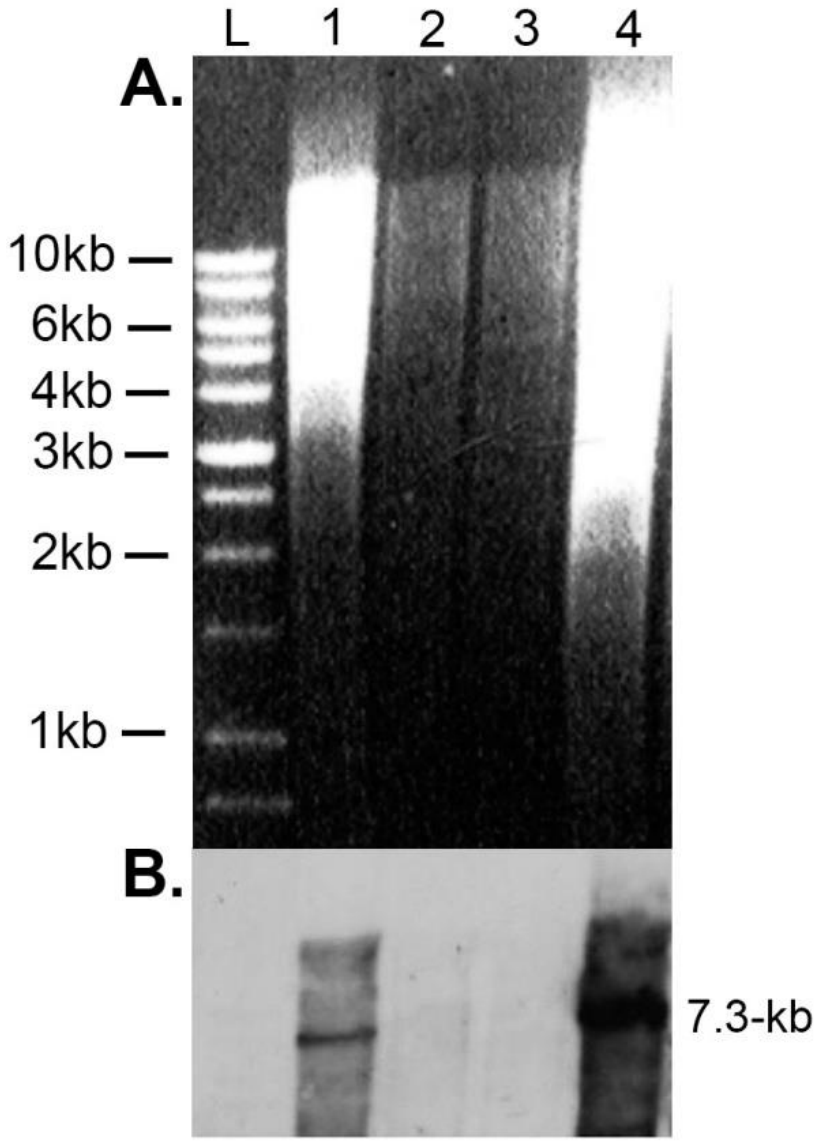

Figure 3.11 Southern blot analysis of the *PKS1* gene complementation. (A). Genomic DNA of strain 3.1 (lane 1), the two Δ*Lppks1*::*hph* mutants corresponding to lane 5 and 7 of the Southern blot shown in Figure 3.8 (lanes 2 and 3), and a putative Δ*Lppks1*::*hph*:PKS complemented strain (lane 4) derived from the Δ*Lppks1*::*hph* mutant shown in lane 7 of the Southern blot in Figure 3.8. DNA was digested with the restriction enzyme *Bg*lII, fractionated by gel electrophoresis and blotted onto Hybond-NX membrane **(B)**. Lane L is DNA size marker. The membrane was probed with a 0.842-kb fragment of the *PKS1* ORF. The presence of a single 7.3-kb band in lane 4 (comparable to lane 1) indicates successful complementation of the Δ*Lppks1*::*hph* mutant (corresponding to lane 7 of Figure 3.8) and production of the complemented strain Δ*Lppks1*::*hph*:PKS.

3.3.5 Pigmentation, hyphal growth and sporulation of Δ*Lppks1*::*hph* and Δ*Lppks1*::*hph*:PKS mutants

Targeted disruption of the polyketide synthase-encoding gene *PKS1* resulted in complete loss of pigmentation in the Δ*Lppks1*::*hph* mutant (Figure 3.12B) when compared to the wild type strain 3.1 (Figure 3.12A). Complementation of the Δ*Lppks1*::*hph* mutant restored melanin production (Figure 3.12C), resulting in a grey phenotype of the ∆*Lppks1::hph:PKS* complementation mutant similar to that of the wild-type strain 3.1. Growth of the fungi on OA revealed that no significant difference in growth rates compared to wild type strain 3.1 (Figure 3.13). Furthermore, there was no significant differences in spore production of the three mutants after 14 days growth on OA (Figure 3.14).

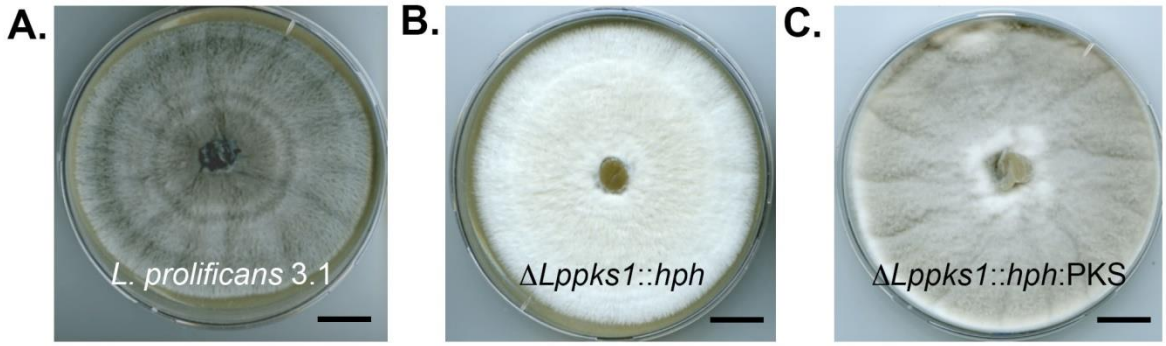

Figure 3.12. Colony morphologies and pigmentations of *L. prolificans* 3.1 and the mutants Δ*Lppks1*::*hph* and ∆*Lppks1::hph*:PKS. (A) Colony morphology of wild-type strain 3.1 after 2-weeks growth on OA at 30 ºC showing typical grey phenotype. **(B)** Morphology of the Δ*Lppks1*::*hph* mutant corresponding to lane 7 of Figure 3.8 after two-weeks growth on OA at 30 ºC. Note the albino phenotype and complete loss of pigmentation. **(C)** Colony morphology of the Δ*Lppks1*::*hph* complemented strain Δ*Lppks1*::*hph*:PKS after 2-weeks growth on OA at 30 °C, showing restoration of melanin production and a grey phenotype similar to that of the wild-type strain 3.1. Scale bars = 1.5 cm.

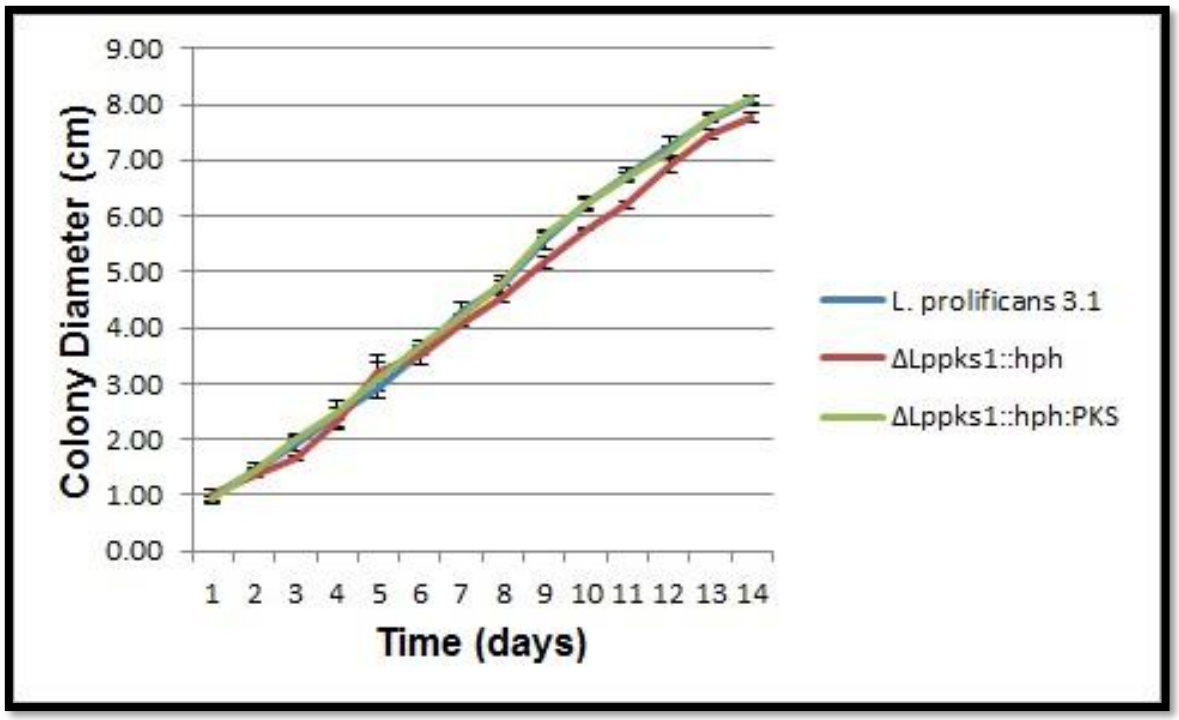

Figure 3.13. Colony diameters of fungi grown on OA for 14 days. By day 14 there was no significant difference in colony diameters of the wild-type strain 3.1, and mutants Δ*Lppks1*::*hph* and Δ*Lppks1*::*hph:PKS* strain (P<0.05; Student's t-test). Each point is the mean of 3 replicates ± standard error.

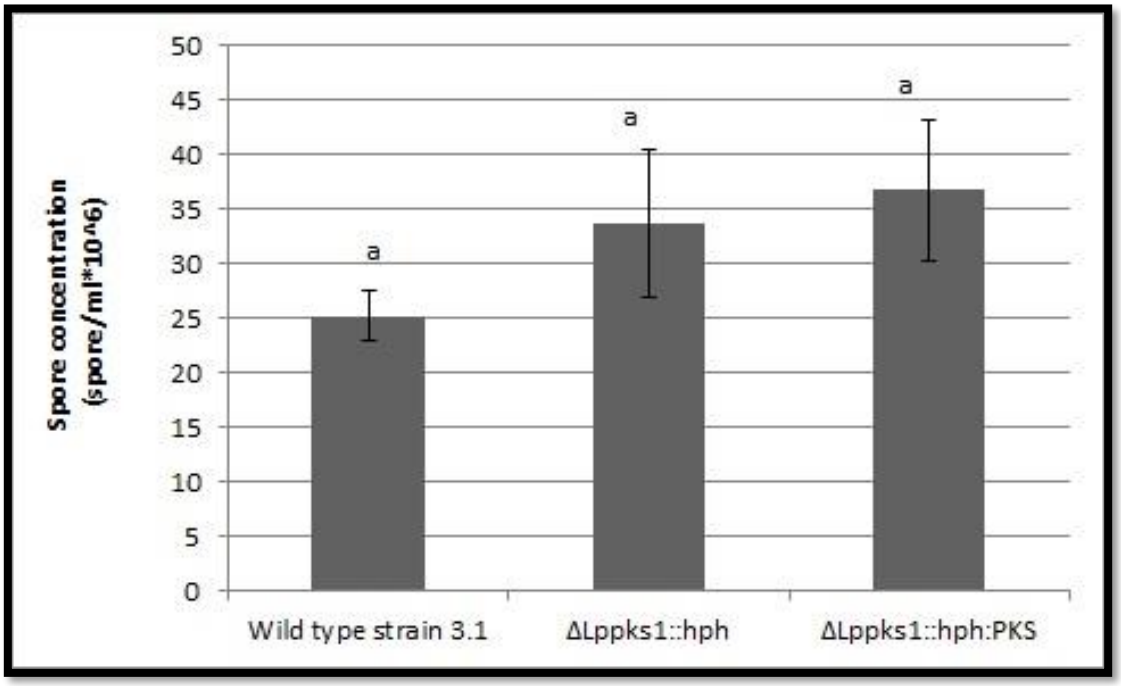

Figure 3.14. Quantification of spore concentrations from 14-day-old OA plate cultures. Results showed no significant difference between the three strains. Each bar is the mean of 3 replicates ± standard error and bars with similar letters indicate that differences in means are not statistically significant at $P<0.05$ (ANOVA).

3.4 Discussion

The aim of this chapter was to disrupt and complement the polyketide synthase (PKS)-encoding gene *PKS1* in *L. prolificans*, and to phenotypically characterise a PKS-deficient mutant Δ*Lppks1*::*hph* and complemented strain Δ*Lppks1*::*hph*:PKS with restored PKS activity. Polyketide synthase converts acetyl CoA + malonyl CoA to tetrahydroxynaphthalene (1,3,6,8-THN) in the first step (Figure 3.1, Step 1) of the DHN-melanin biosynthesis pathway in fungi (Webster, et al., 2007). Loss of PKS activity in *L. prolificans* would result in its inability to produce 1,3,6,8-THN, the substrate of the enzyme tetrahydroxynaphthalene reductase (4HNR) and which, along with the enzyme scytalone dehydratase (SCD), catalyse subsequent reactions (Figure 3.1, Steps 2 and 3, respectively) in the melanin biosynthesis pathway (the subject of Chapter 4).

Cloning, sequencing and alignment of the *PKS1* gene confirmed its close homology with PKS-encoding genes in other fungi, while the *L. prolificans* whole genome sequence generated in-house provided sufficient coverage and quality to allow its targeted deletion in the *L. prolificans* wild type strain 3.1 (Thornton, *et al*., 2015b). Targeted deletion of the *L. prolificans PKS1* gene was carried out by replacing the open reading frame of the gene with the gene *hph* (conferring resistance to the antibiotic hygromycin B), following the split marker technique of homologous recombination. This resulted in the successful generation of two Δ*Lppks1*::*hph* mutant strains. Many ectopic hygromycin-resistant transformants were also generated suggesting that homologous recombination events in *L. prolificans* are rare, an observation made in other fungi such as *Cryptococcus neoformans* (Goins, *et al*., 2006; Kück, *et al*., 2010). Several studies have demonstrated that prior disruption of the Non-Homologous End Joining (NHEJ) system can decrease the number of ectopic integrations in fungi (Goins, *et al*., 2006; Takahashi, *et al*., 2006; Kück, *et al*., 2010), and a similar approach could be used in *L. prolificans* to improve the likelihood of homologous recombination in this pathogen.

The frequency of homologous recombination is also influenced by the length of the flanking regions on either side of the open reading frame (ORF). In the yeast *Saccharomyces cerevisiae*, short homologous sequences of approximately 30-bp upstream and downstream of the ORF are adequate to achieve successful

homologous recombination (Hua, *et al*., 1997; Kück, *et al*., 2010). In filamentous fungi, increasing the size of the flanks improves the likelihood of homologous recombination (Hua, *et al*., 1997), and increases transformation efficiencies (You, *et al*., 2009). For these reasons, the lengths of the flanking regions chosen here for disruption of the *PKS1* gene were ~1.920-kb (left flank) and ~1.913-kb (right flank) of the ORF owing to the large size of the ORF of ~6.708-kb. Due to the large size of the *PKS1* ORF, complementation of the Δ*Lppks1*::*hph* mutant was also only achieved using the In-Fusion® HD Cloning Kit. The kit provides a high efficiency of cloning of large DNA fragments, that is not achievable using standard cloning methodologies.

The large ORF of the PKS1-encoding gene was accounted for by multiple domains comprising ketoacyl-synt-C, ketoacyl-syn, acyl transferase, polyketide synthase dehydratase, phosphopantetheine attachment site, and thioesterase domains, which identified the *L. prolifcans* enzyme as a Type I polyketide synthase (Takano, *et al*., 1995; Feng, *et al*., 2001). Of the three types of PKS, Type I enzymes are involved in the synthesis of fungal DHN-melanin (Takano, *et al*., 1995).

The PKS-deficient strains Δ*Lppks1*::*hph* generated here had an albino phenotype, indicating complete loss of melanin production. This is consistent with the disruption of the *PKS1* genes in the fungi *Bipolaris oryzae* and *Aspergillus fumigatus* (Tsai, *et al*., 1998 ; Moriwaki, *et al*., 2004), which similarly have albino phenotypes due to blocks in the first steps of DHN-melanin biosynthesis. The *L. prolificans* albino mutants Δ*Lppks1*::*hph* and complemented strain Δ*Lppks1*::*hph*:PKS showed no significant differences in hyphal growth and sporulation when compared to the wild-type strain 3.1. This is consistent with the finding of Wange and co-workers (Wang, *et al*., 2010) with PKS-deficient albino mutants of *Grosmannia clavigera*.

In summary, work undertaken in this chapter has led to the successful development of albino mutants of *L. prolificans* that lack melanin production due to disruption of the polyketide synthase-encoding gene *PKS1*. Re-introduction of the gene into the enzyme-deficient mutant Δ*Lppks1*::*hph* resulted in a complemented strain Δ*Lppks1*::*hph*:PKS with restored melanin production and grey colouration similar to the wild-type. Alongside mutants described in Chapter 4 that are deficient in the melanin biosynthetic enzymes scytalone dehydratase and tetrahydroxynaphthalene

reductase, the consequences of targeted disruption of *PKS1* for pathogen survival can investigated, and are reported in Chapters 5 and 6.

Chapter 4

Targeted deletion and complementation of the scytalone dehydratase-encoding gene *SCD1* in *Lomentospora prolificans*

4.1 Introduction

4.1.1 Scytalone dehydratase and DHN melanin biosynthesis

Scytalone dehydratase (SCD; EC4.2.1.94) is an enzyme involved in dihydroxynaphthalene (1,8-DHN)-melanin biosynthesis, catalysing the chemical reaction scytalone ⇆ 1,3,8-trihydroxynaphthalene + H_2O (Figure 4.1). The substrate scytalone is first produced from 1,3,6,8-THN by the enzyme tetrahydroxynapthalene reductase (4HNR). The production of a 4HNR-deficient mutant of *L. prolificans* (Δ*Lp4hnr*::*hph*) has been reported by us previously (Thornton, *et al*., 2015b), and is included here for comparative phenotypic analysis alongside the SCD-deficient mutant developed and described in this chapter.

Scytalone dehydratase is a symmetric trimer, composed of three analogous single-domain subunits (Lundqvist, *et al*., 1994). The conformation of each subunit has been described as cone-shaped and is composed of both an α and β barrel, with an interior hydrophobic active site (Lundqvist, *et al*., 1994). To study the catalysis and substrate binding mechanisms of SCD in the ascomycete *Magnaporthe grisea*, strains with mutations in the enzyme's active site were generated (Basarab , *et al*., 1999). It was shown that all active site amino acid residues (Asp31, Asn131, Tyr50, Tyr30, His110, His85, Lys73 and Ser129) are necessary in substrate binding and catalysis with the exception of Lys73 (Basarab , *et al*., 1999). During dehydration, an intermediate product (enolate) stabilizes the reaction by contributing water and an internal hydrogen bond (Zheng, *et al*., 1998). The roles of the tyrosine residues Tyr-50 and Tyr-30 are to promote the protonation of substrate carbonyl via water (Zheng, *et al*., 1998).

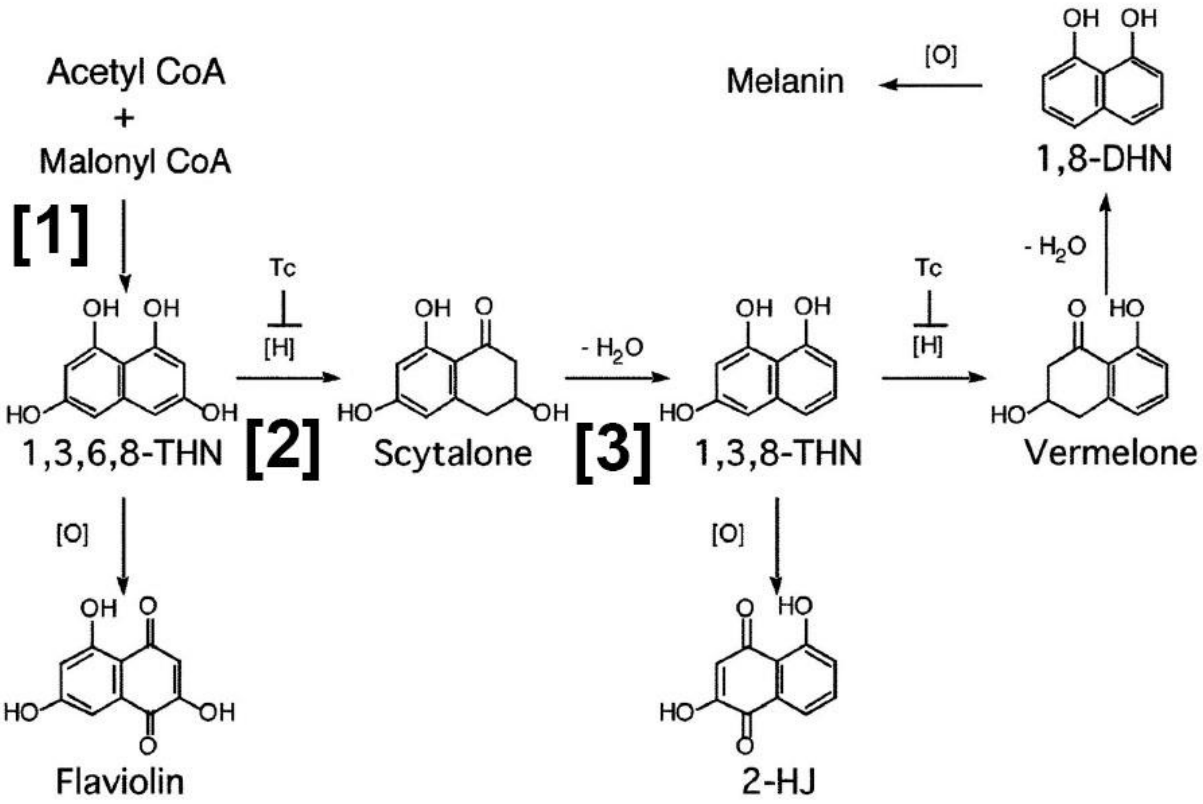

Figure 4.1. Schematic diagram showing the DHN melanin biosynthetic pathway in *L. prolificans*. Step (3) scytalone to 1,3,8-trihydroxynaphthalene (1,3,8-THN) is catalysed by the enzyme scytalone dehydratase, while steps (1) and (2) are catalysed by the enzymes polyketide synthase (Chapter 3) and tetrahydroxynaphthalene reductase (Thornton et al. 2015b and this chapter), respectively. Taken from Al-Laaeiby *et al*. (2016).

Scytalone dehydratase genes and their enzyme products have been characterised in the fungi *Colletotrichum lagenarium*, *Magnaporthe grisea* and *Ophiostoma floccosum* (Kubo, *et al*., 1989; Lundqvist, *et al*., 1994; Wang , *et al*., 2001; Fleet, *et al*., 2002). The gene in *Colletotrichum lagenarium*, a plant pathogen which infects cucumber leaves, encodes a protein of 188 amino acids in length (Kubo, *et al*., 1996). Defects in the *Colletotrichum lagenarium* gene result in the production of reddish-brown mutants (Kubo, *et al*., 1996), whereas an *arp1* gene deficient mutant of *Aspergillus fumigatus* generated by molecular genetic approaches often reddish-pink conidia (Tsai , *et al*., 1997). Some authors demonstrated that these mutants accumulate scytalone, an intermediate of DHN-melanin biosynthesis (Kubo, *et al*., 1983; Tanaka, *et al*., 1991; Wang, *et al*., 2010). Re-introduction of the scytalone dehydratase-encoding gene into enzyme-deficient mutants of *Cochliobolus heterostrophus* and *Colletotrichum lagenarium* restores melanisation and, in *Colletotrichum lagenarium*, restores pathogenicity of the fungus and its ability to penetrate host leaves (Kubo, *et al*., 1996 ; Saitoh, *et al*., 2012).

The involvement of SCD in the pathogenicity of the rice blast pathogen *Magnaporthe grisea* has also been shown, with melanin providing the appressorial rigidity needed to penetrate plant cells (Talbot, 2003). Scytalone dehydratase is a target of inhibitors such as carpropamid, and carpropamid-resistant strains recovered in Japan (Tsuji, *et al*., 1997) were shown to have point mutations, causing a single amino acid substitution of valine to methionine in their enzyme-encoding genes (Takagaki, *et al*., 2004).

The gene *Sal1* gene is responsible for scytalone dehydratase production in *Cochliobolus heterostrophus* (Saitoh, *et al*., 2012). Cloning and characterization of the *Sal1* gene shows that the open reading frame (ORF) comprises 552 bp and shares 49% identity with *ARP1*, 98% identity with *SCD1*, 79% identity with *SCD1*, and 74% identity with *OSD1* of *Aspergillus fumigatus, Bipolaris oryzae, Colletotrichum orbiculare and Ophiostoma floccosum* respectively (Saitoh, *et al*., 2012). The Arp1 protein sequence of *Aspergillus fumigatus* shares 75% and 76% similarity with the scytalone dehydratases of *Colletotrichum lagenarium* and *Magnaporthe grisea* respectively (Tsai , *et al*., 1997). The *ARP1* gene in *Aspergillus fumigatus* encodes a 168 amino acid protein and the gene is interrupted by two introns (Tsai , *et al*., 1997). Disruption of the *Aspergillus fumigatus ARP1* gene increased the deposition of the

soluble complement component C3 on the conidial surface (Tsai, *et al*., 1997). Characterisation of the SCD1 gene of *Bipolaris oryzae* shows that it encodes a putative protein of 185 amino acids in length (Kihara, *et al*., 2004b), while RNA analysis with Northern blotting shows enhanced transcription when the fungus is exposed to near-violet (300-400 nm) radiation (Kihara, *et al*., 2004a).

Disruption of the scytalone dehydratase-encoding gene in *Grosmannia clavigera* leads to accumulation of scytalone and a change in colony colour from black in the wild-type to a reddish pigmentation in mutants (Wang, *et al*., 2010). Wang and co-workers confirmed the accumulation of scytalone in the scytalone dehydratase-deficient mutants using plate challenge assays with a polyketide synthase-deficient (albino) mutant of the same fungus. On contact, the albino mutant converted to black, indicating the accumulation of scytalone in the scytalone dehydratase-deficient mutant. The albino mutant was able to complete DHN-melanin biosynthesis since it was intact for scytalone dehydratase, converting scytalone to 1,3,8-DHN (Wang*, et al.,* 2010*).*

4.1.2 Aims of Chapter 4

In this chapter, I set out to generate a mutant of *L. prolificans* deficient in the DHN-melanin enzyme scytalone dehydratase (SCD) and to compare its phenotype to a previously developed mutant of the fungus (Δ*Lp4hnr*::*hph*) deficient in the enzyme tetrahydroxynaphthalene reductase (4HNR), which is unable to produce the SCD substrate scytalone. To this end, I describe the use of a split-marker homologous recombination method for generation of the SCD-deficient mutant and attempts to re-introduce the enzyme-encoding gene through complementation. The phenotype (growth habit, spore production, pigmentation) of the Δ*Lpscd1*::*hph* mutant is compared to that of the enzyme-deficient mutant Δ*Lp4hnr*::*hph*, while the consequences of gene deletion and loss of normal melanisation to the survival of the mutants is described in Chapters 5 and 6.

4.2 Material and Methods

4.2.1 Multiple DNA sequence alignments

The DNA sequence of the scytalone dehydratase-encoding gene *SCD1* was obtained by interrogation of the *L. prolificans* strain 3.1 (Thornton, *et al*., 2015b) full genome sequence archived at Biosciences, University of Exeter. The programme Basic Local Alignment Search Tool (BLAST) was used to investigate the sequence homologies between the ORF of the *SCD1* gene of *L*. *prolificans* and *SCD* gene sequences from other species deposited in public databases (*Colletotrichum musae, Podospora anserina, Marssonina brunnea, Sporothrix schenckii* and *Penicillium marneffei*). Sequence alignments were constructed by using DNAMAN software (http://www.lynnon.com/dnaman.html), which identifies identical, similar and non-identical nucleotides.

4.2.2 Primer design

The full genome sequence of *L. prolificans* strain 3.1 archived at Biosciences, University of Exeter was used to design primers for disruption and complementation of the gene *SCD1*, and are shown in Table 4.1. Primers were constructed by using the online resource (http://depts.washington.edu/bakerpg/primertemp/primermelttemp.html) and for reverse complements (http://arep.med.harvard.edu/labgc/adnan/projects/Utilities/revcomp.html).

Table 4.1. Details of primer sequences used in the disruption and complementation of the *L. prolificans* SCD-encoding gene *SCD1*.

Primer Name	Sequence 5'-3'	Product
Lpscd1-F	GATCGCCATCCCAGCCATCA	*Lpscd1* ORF
Lpscd1-R	ACGTGGCAAGGGTTGGATCC	*Lpscd1* ORF
Lpscd1-LFF	CGTCAGCTTTGGAAAACAAC	*Lpscd1* LF
Lpscd1-LFR	GTCGTGACTGGGAAAACCCTGGCG ACGGTAGCCATGTTTTCCGA	*Lpscd1* LF
Lpscd1-RFF	TCCTGTGTGAAATTGTTATCCGCTTATCT GGCGTCAATCGGAAA	*Lpscd1* RF
Lpscd1-RFR	ACATGTTGTTTTGCACGCTT	*Lpscd1* RF
HY split	GGATGCCTCCGCTCGAAGTA	*Lpscd1* LF + HY
YG split	CGTTGCAAGACCTGCCTGAA	*Lpscd1* YG + RF
M13F	CGCCAGGGTTTTCCCAGTCACGAC	-
M13R	AGCGGATAACAATTTCACACAGGA	-
Lpscd1-f1	TAGAACTAGTGGATCATAATCCCCACCT TTCGACA	*Lpscd1* gene fragment
Lpscd1-r1	CGGTATCGATAAGCTAATCGATTCGGCC GTTTCTT	*Lpscd1* gene fragment

The reverse complement of M13 forward and reverse sequences, used with HY split and YG split respectively, are shown underlined. ORF: open reading frame; LF: left flank; RF: right flank; LFF: left flank forward; LFR: left flank reverse; RFF: right flank forward; RFR: right flank reverse.

4.2.3 Targeted disruption of the *SCD1* gene and generation of Δ*Lpscd1*::*hph* mutant

Targeted gene disruption was carried out following the split marker method (Yu et al., 2004) using the protoplast transformation procedure outlined in Section 2.3. For targeted replacement of the SCD-encoding gene *SCD1*, two rounds of PCR were required to complete the process (Figure 4.2). In the first round PCR, the left (upstream)(0.963-kb) and right (downstream)(0.922-kb) flanking regions of the *SCD* ORF were amplified from *L. prolificans* genomic DNA (Section 2.2.1) using primer pairs Lpscd1-LFF/Lpscd1-LFR and Lpscd1-RFF/Lpscd1-RFR designed to include an extension complementary to the hygromycin phosphotransferase gene (*HPH*) conferring resistance to the antibiotic hygromycin B (Table 4.1). The left and right fragments of the *HPH* gene were amplified from pCB1004 with primer pairs HY split/M13F and M13R/YG split, respectively. In a second round of PCR, the left flank was fused with one half of the hygromycin cassette (HY)(1.184-kb) and the right flank with the other half (YG)(0.773-kb). The second round PCR products LF+HY (2.147-kb) and RF+YG (1.695-kb) were used for protoplast transformation (Section 2.3). Homologous recombination resulted in the replacement of the *SCD1* ORF with the functional *HPH* gene. Putative ∆*Lpscd1*::*hph* mutants were selected based on resistance to 200 µg/ml of the antibiotic hygromycin B (Section 2.3).

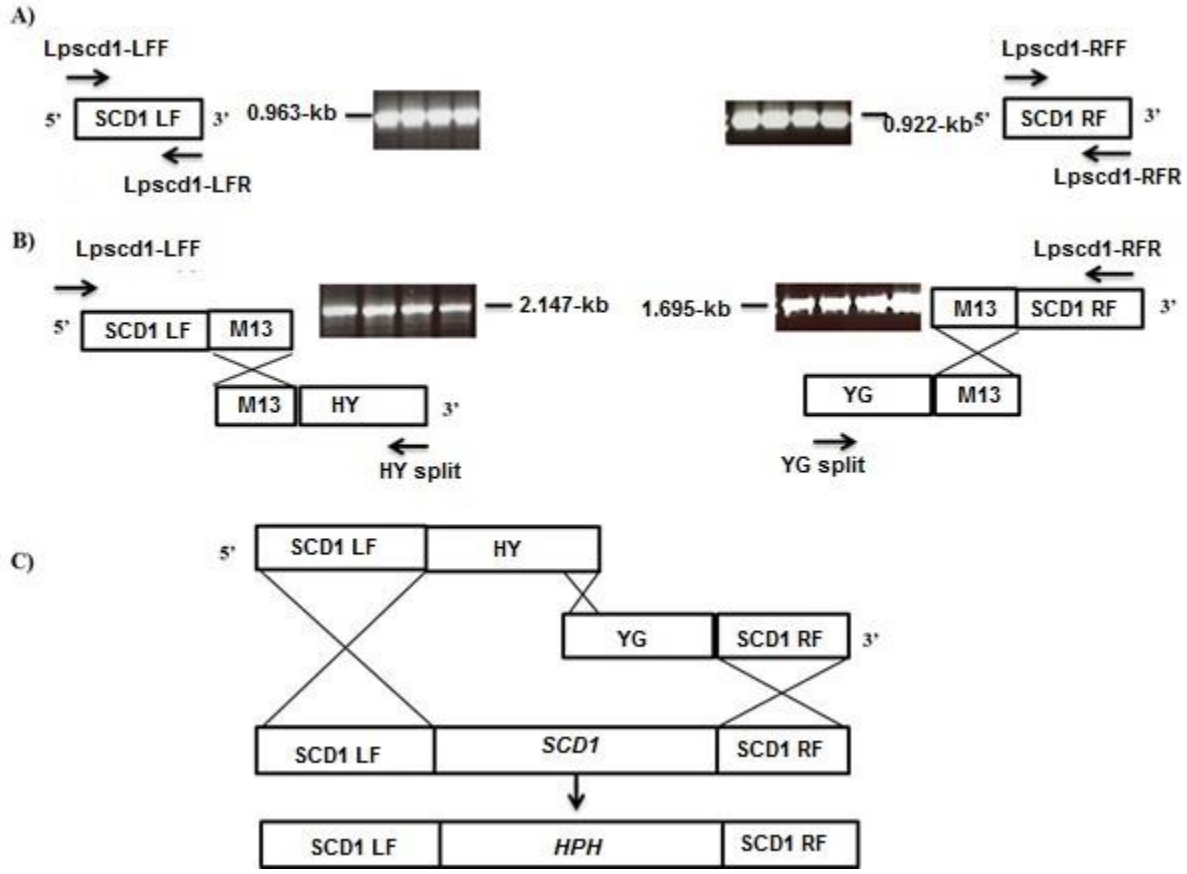

Figure 4.2. Schematic representation of the targeted gene replacement strategy for the SCD-encoding gene *SCD1*. A) First round PCR: the left (upstream)(0.963-kb) and right (downstream)(0.922-kb) flanking regions of the *SCD1* ORF were amplified using the primers shown. **B)** Second round PCR was carried out to fuse the flanks of *SCD1* with overlapping fragments of the hygromycin cassette (HY and YG). **C)** Homologous recombination event in which the *HPH* cassette replaces the *SCD1* ORF and is integrated into the sequence, thereby disrupting the SCD-encoding gene.

4.2.4 Southern blotting

Hygromycin B resistant mutants were investigated further by Southern blot analysis (Section 2.4). Genomic DNA from putative Δ*Lpscd1*::*hph* mutants and the wild-type strain 3.1 were digested with the restriction enzyme *Bgl*II according to the methods outlined in Section 2.2.2.1. The products were separated by agarose gel electrophoresis and blotted onto a Hybond-NX membrane (Section 2.4). The membrane was probed with a 0.963-kb fragment upstream of the *SCD1* ORF. Putative Δ*Lpscd1*::*hph* mutants were identified by a fragment size of 6.9-kb compared to the 5.7-kb fragment of the wild-type strain.

4.2.5 Complementation of the Δ*Lpscd1*::*hph* mutant

The Δ*Lpscd1*::*hph* mutant was complemented by integration of a 3.743-kb fragment consisting of 2.413-kb of promoter region, 0.73-kb of the ORF and 0.6-kb terminator region of the *SCD1* gene to restore gene functionality and concomitant melanin biosynthesis. A schematic diagram illustrating the complementation procedure is shown in Figure 4.3. The *SCD1* fragment was amplified by PCR using the gene-specific primers Lpscd1-f1 and Lp-scd1-r1 (Table 4.1), designed to include 15-bp of sequence homologous to the ends of the linearized vector pCB1532. The vector PCB1532, which contains the sulfonylurea resistant allele of the *Magnaporthe oryzae ILV1* gene (Sweigard, et al., 1997), was linearized digested by with the restriction enzymes *Bam*HI and *Hin*dIII. The PCR product was ligated to linearised pCB1532 vector and used to transform competent *E. coli* cells (Section 2.2.2.4.2), which were selected based on white-blue screening (Section 2.2.2.4.2). Plasmid DNA was purified (Section 2.2.2.4.4) and sequenced to confirm the correct *SCD1* insertion and the vector then transformed into the mutant Δ*Lpscd1*::*hph*. Putative Δ*Lpscd1*::*hph*:SCD1 complementation mutants were selected based on resistance to 100 µg/ml sulfonylurea.

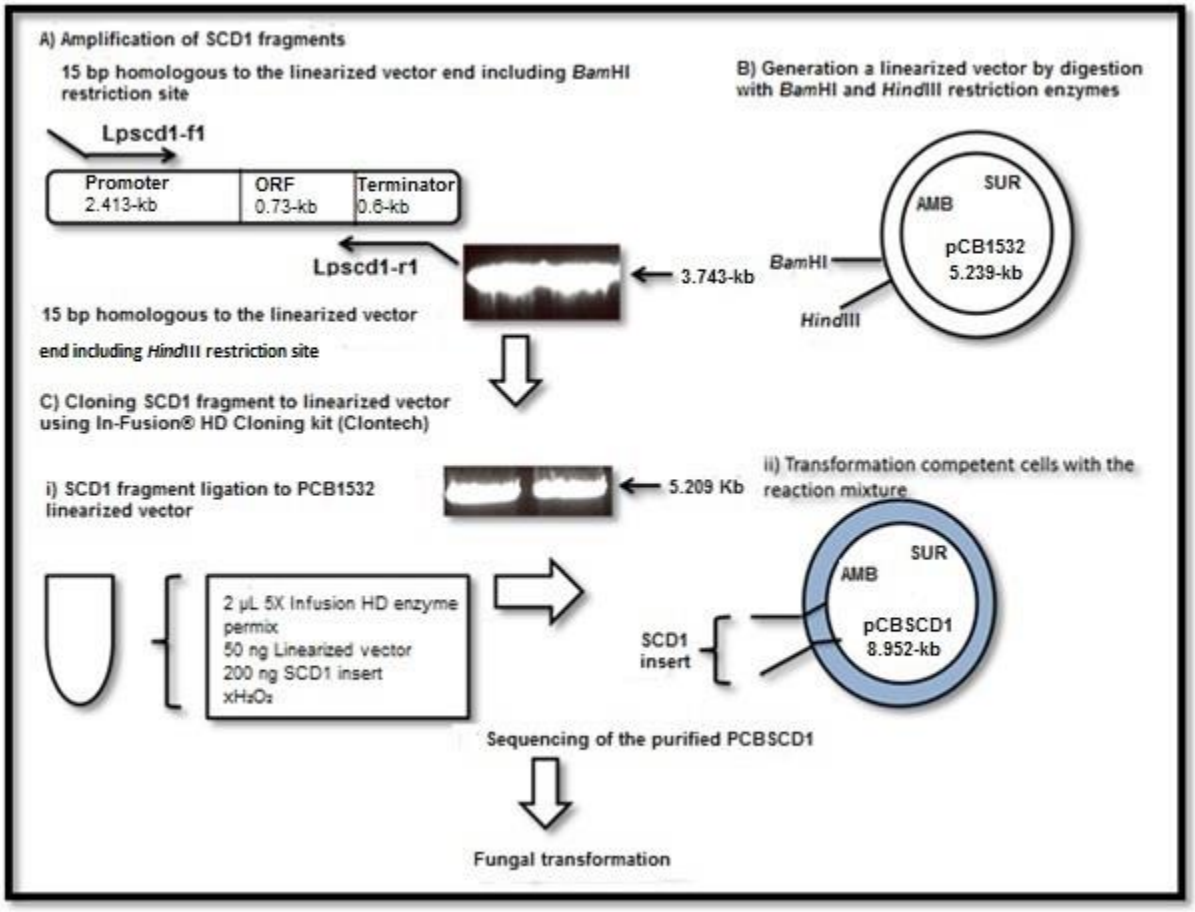

Figure 4.3. Schematic diagram showing procedure for complementation of the Δ*Lpscd1*::*hph* mutant. A) Amplification of the 3.743-kb fragment of the *SCD1* gene consisting of promoter region (2.413-kb), ORF (0.73-kb) and terminator (0.6-kb), using PCR primers designed to include 15-bp of sequence homologous to the ends of the linearised vector pCB1532. **B)** Digestion of pCB1532 with *Hin*dIII and *Bam*HI restriction enzymes to create the linearised vector. **C)** Cloning of the *SCD1* fragment to the linearized pCB1532 vector; the *SCD1* fragment is ligated to the vector and the reaction mixture used to transform Stellar competent cells. Once confirmed by sequencing, the vector is used to transform fungal protoplasts for generation of complemented mutants.

4.2.6 Hyphal growth, sporulation and pigmentation

Stable transformants were generated following three rounds of single spore isolation under antibiotic selection (hygromycin or sulfonylurea). Spores were harvested as described (Section 2.2.1) and spore suspensions serially diluted before spreading on CM/BDCM selection plates and incubation for 3 days at 30 ^{o}C in the dark. Colonies developing from single spores were sub-cultured and the process repeated twice more. For hyphal growth determinations, strains were inoculated centrally onto replicate OA plates and colony diameters were measured over a 2-wk growth period at 30 ^{o}C, with spore production quantified after 14 d. Spores were suspended in 20 ml dH_2O using plastic L-shaped spreaders, filtered through Miracloth, and spore concentrations determined using a haemocytometer. There were 3 replicates for each experiment and experiments were repeated 3 times.

4.3 Results

4.3.1 *SCD1* gene sequence and homology to other fungal *SCD1* genes

The nucleotide sequence of the *SCD1* gene recovered from the *L. prolificans* 3.1 whole genome sequences is shown in Figure 4.4. The ORF of the *L. prolificans SCD1* gene is 730-bp long and has a single intron at 180-334 bp (highlighted yellow in Figure 4.4). The ORF encodes a protein of 162 amino acids (Figure 4.5).

The gene sequence was deposited in the NCBI database, with the accession number (KY646315).

GCCCACCCTCCCGGCCCCCGAGCCTTTCACCAGATGCTTCAGCACGAGGGATGCATCCCA
CCCGTCGTCTCAGCTGCCCAAGAAACGGCAAGTACTATTTGTTTGGCAGCTGCTGGTTTT
TCCTACTTTCTTTTTGGTCGTTCCCTTCCATCCCGCCCCGCGAACCACTCTTCCCACATA
GTACGTGGGGAACGTGAAACTCCTCTCCGCTTCGGTTCAGCGTCGGCGTTGGATCCGGGA
GAACGGCTCCGTCTGGTGCGGGATAGGTTGTCTTTGGGGACCAATACCAGATCTTGTCTT
CGTTTGGCTCACCACTCTCTTTCATATGTTATGCCTCCCGAGGCGGCATGTGCGTCTGAG
AATGCGGAGAGCCAAAAGATAATACCACCTTCTGCCATGCTTGCGCACGTCCTTTTCCCT
ACACAAATCTTGCTTAGGGTGAAAAGAGGTATTGCCTGGCGAGGGTCAAGGGTGGTGGCC
TCAGCGAAATGACGAGGGTAGTCGCTGGCAGTAGCCAGCTTGCAGGGTCACTAATAGTGG
GTCTAGCCCCATATCAGCCAGCGTCCCCTCCTCCTTAATTGAGTGCATGGGGAATGTGCT
GGTCTGAAGAGGTACAACTCATCGTACCTTGCAGCCTGGGGCTCATTCCCCATTCCCCCC
ATCGCCTGGCAGACGAGGAAGAGTAATATTGCCTTCGTTTCGTCTATGATTGCCAACAGT
TAGCTGCTCCGCCATCCTTCCCCCTTGCCTATTACTGCTGGCTCTCCCTTCTCTCGATCC
CCGCTAGCCCCAACTCCCATTCTCCGTTTCTTGTTTGACGTGTTCATTTCTAAATGCCGA
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TTGCGATTGCAATTTTTAGAACCCCCCACATTCA

Figure 4.4. Nucleotide sequence of the *L. prolificans* scytalone dehydratase-encoding gene *SCD1* with flanking regions. The open reading frame of the gene is shown in bold type and highlighted in grey and the start (ATG) and stop (TGA) codons are underlined. The intron is highlighted in yellow. The Ns highlighted in red indicate a sequence error.

MHPNFFTHAQEWRDPSNARGGIPQTLTDYLGLNAALYEWADSYDSKDWTRLSKCIAPTLRIR
SFLDKMWEAMPAQEFLAMISNPSVLGDPTLKTQHFVGASRWERISADEVVGYHQLRVPHQRY
DRDPAQQGAKVTAKGHAHSANKHWYRKVNGVWKFAGLCPDIRWSEYDFDKIFAGGRENFGEN
GQQ

Figure 4.5. The protein sequence of the *L. prolificans* scytalone dehydratase enzyme following translation of the *SCD1* gene. The single domain, located between amino acids 24-161, is highlighted in yellow.

The program Basic Local Alignment Search Tool (BLAST) was used to investigate homology of the *SCD1* gene with comparable gene sequences deposited in public database. The ORF of the *L. prolificans SCD1* gene (Figure 4.4) when aligned against other ORFs (Figure 4.6), was shown to have 72% homology with *Podospora anserina*, 89% with *Marssonina brunnea*, 80% with *Sporothrix schenckii*, 91% with *Penicillium marneffei* and 77% with *Colletotrichum musae.* Using DNAMAN software, identical nucleotides are shown in blue, similar nucleotides are shown in pink, while non-identical nucleotides are unshaded.

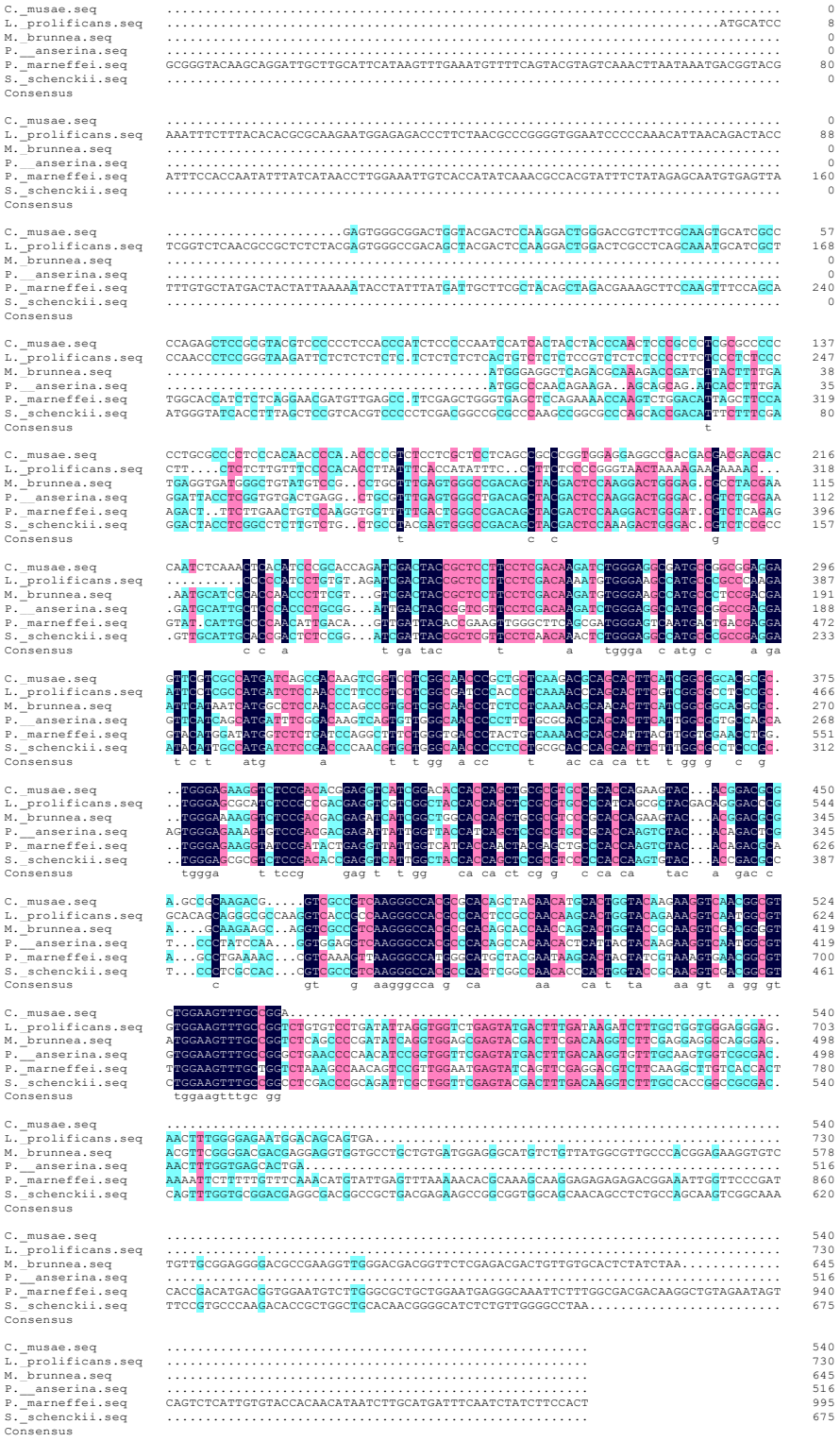

Figure 4.6. Nucleotide sequence alignment of the *L. prolificans SCD1* gene against comparable SCD-encoding gene sequences from *Penicillium marneffei*, *Sporothrix schenckii*, *Colletotrichum musae*, *Marssonina brunnea* and *Podospora anserina*. Identical nucleotides are shown in blue, similar nucleotides in pink and non-identical sequences are un-shaded.

4.3.2 Generation of Δ*Lpscd1*::*hph* mutant

Putative transformants were identified following re-generation of transformed protoplasts in the presence of 200 µg/ml of the antibiotic hygromycin B. Figure 4.7 shows growth of a putative mutant following protoplast re-generation and sub-culture onto Complete Medium containing 200 µg/ml hygromycin.

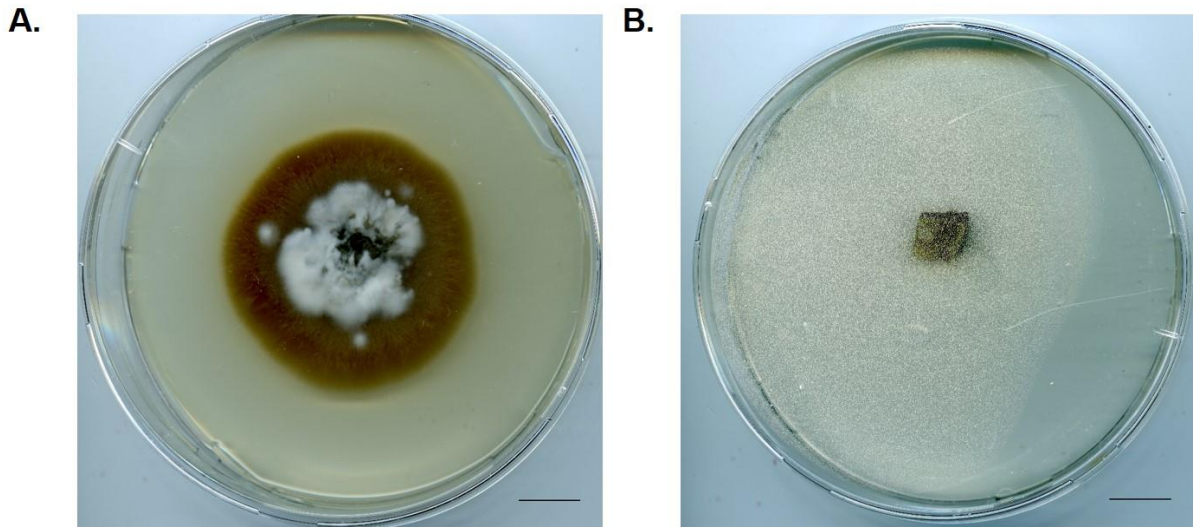

Figure 4.7. Growth of the wild-type *L. prolificans* strain 3.1 and a putative Δ*Lpscd1*::*hph* mutant on complete medium (CM) supplemented with 200 µg/ml hygromycin and grown at 30°C for 7 days. Note growth of the putative mutant **(A)**, but lack of growth of the wild-type strain **(B)**. Image was captured using an Epson Expression 1680 Pro scanner. Scale bars = 1.5 cm.

4.3.3 Southern blotting

Using Southern blotting, a single Δ*Lpscd1*::*hph* mutant was identified. Genomic DNA of the wild-type strain 3.1 and a single hygromycin-resistant strain, digested with the restriction enzyme *Bg*III (Figure 4.8A) and probed with a 1.0-kb fragment upstream of the *SCD1* ORF, showed a single 6.9-kb band compared to the 5.7-kb in the wild-type strain 3.1(Figure 4.8B). The increased size of the band indicates successful replacement of the SCD-encoding gene in the Δ*Lpscd1*::*hph* mutant.

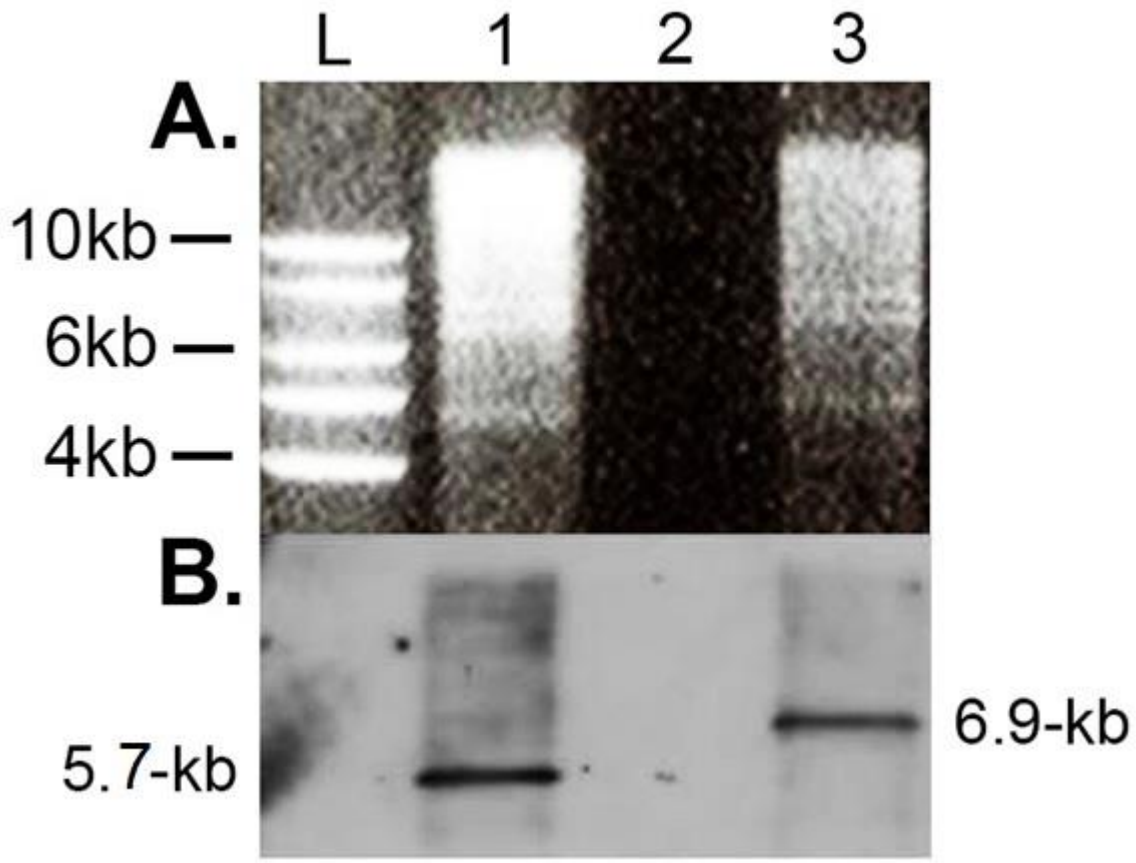

Figure 4.8. Southern blot analysis of the targeted gene replacement of *SCD1*. (A) Genomic DNA of the *L. prolificans* wild-type strain 3.1 (lane 1) and the putative transformant Δ*Lpscd1*::*hph* (lane 3) were digested with the restriction enzyme *Bgl*II, fractionated by gel electrophoresis and blotted onto Hybond-NX membrane. Lane L contains DNA size marker and lane 2 was left blank. **(B)** The membrane was probed with a 1.0-kb fragment upstream of the *SCD1* ORF. The presence of the single 6.9-kb band in lane 3, compared to the single 5.7-kb band in lane 1, indicates successful replacement of the *SCD1* gene.

4.3.4 Pigmentation, hyphal growth and sporulation of the Δ*Lpscd1*::*hph* mutant

Targeted disruption of the melanin biosynthesis gene *SCD1* resulted in abnormal pigmentation in the Δ*Lpscd1*::*hph* mutant when compared to the wild-type strain 3.1 (Figure 4.9). The wild-type 3.1 strain is grey in contrast to the beige colour of the Δ*Lpscd1*::*hph* mutant, while the tetrahydroxynaphthalene reductase-deficient mutant ∆*Lp4hnr1::hph* mutant (Figure 4.9), generated in a previous study (Thornton, et al., 2015b), is yellow-grey. Growth of the fungi on OA revealed no significant differences in growth rates compared to strain 3.1, with the exception of mutant ∆*Lp4hnr*::*hph*. With this mutant, there was a significant reduction in colony diameter by day 14 when compared to the mutant Δ*Lpscd1*::*hph*, and also to the wild-type strain (Figure 4.10), consistent with previous findings (Thornton, et al., 2015b). Spore production in the 14-d-old cultures was significantly different between strain 3.1 and the two mutants (Figure 4.11). There was a significant reduction in spore production in the mutant ∆*Lp4hnr*::*hph,* while spore production of ∆*Lpscd1*::*hph* was significantly increased when compared to the wild-type strain.

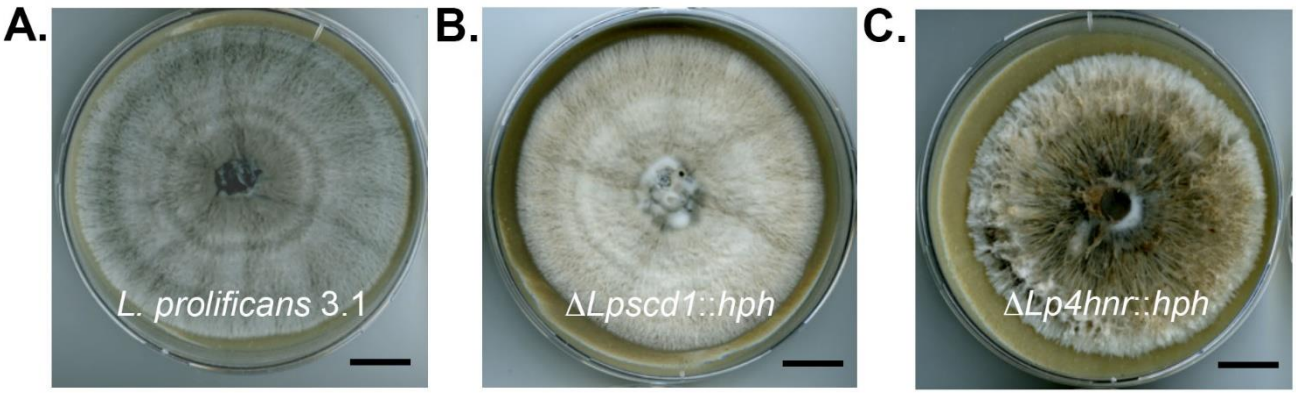

Figure 4.9. Colony morphologies and pigmentations of *L. prolificans* 3.1 and the mutants Δ*Lpscd1*::*hph* and Δ*Lp4hnr*::*hph*. (A). Colony morphology of strain 3.1 after 2-wk growth on OA at 30 ^{o}C showing typical grey phenotype. **(B)**. Morphology of mutant Δ*Lpscd1*::*hph* after 2-wk growth on OA at 30^{o}C showing abnormal beige pigmentation, and comparison to the yellow-grey tetrahydroxynaphthalene reductase-deficient mutant Δ*Lp4hnr*::*hph* **(C)** developed previously (Thornton, et al., 2015b). Images were captured using an Epson Expression 1680 Pro scanner. Scale bars = 1.5 cm.

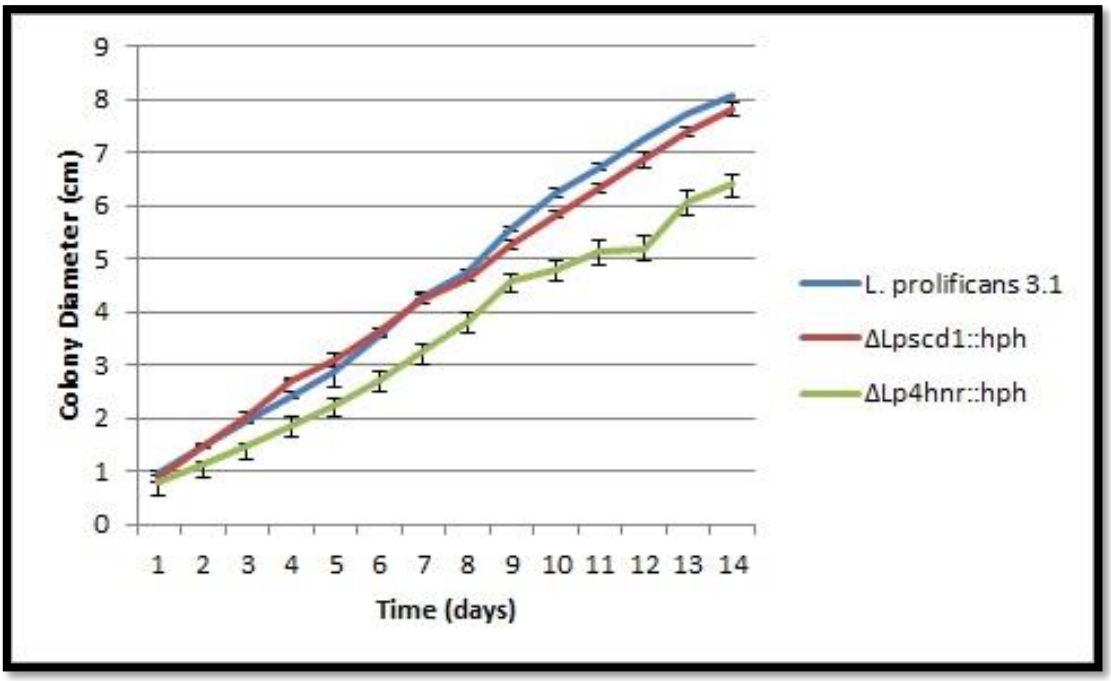

Figure 4.10. Colony diameters of fungi grown on OA for 14 days. By day 14, the mutant Δ*Lp4hnr*::*hph* had significantly reduced growth when compared to the wild-type strain ($P<0.05$; Student's *t*-test). In contrast, the mutant Δ*Lpscd1*::*hph* showed growth comparable to strain 3.1 over the 14 day growth period. Each point is the mean of 3 replicates ± standard error.

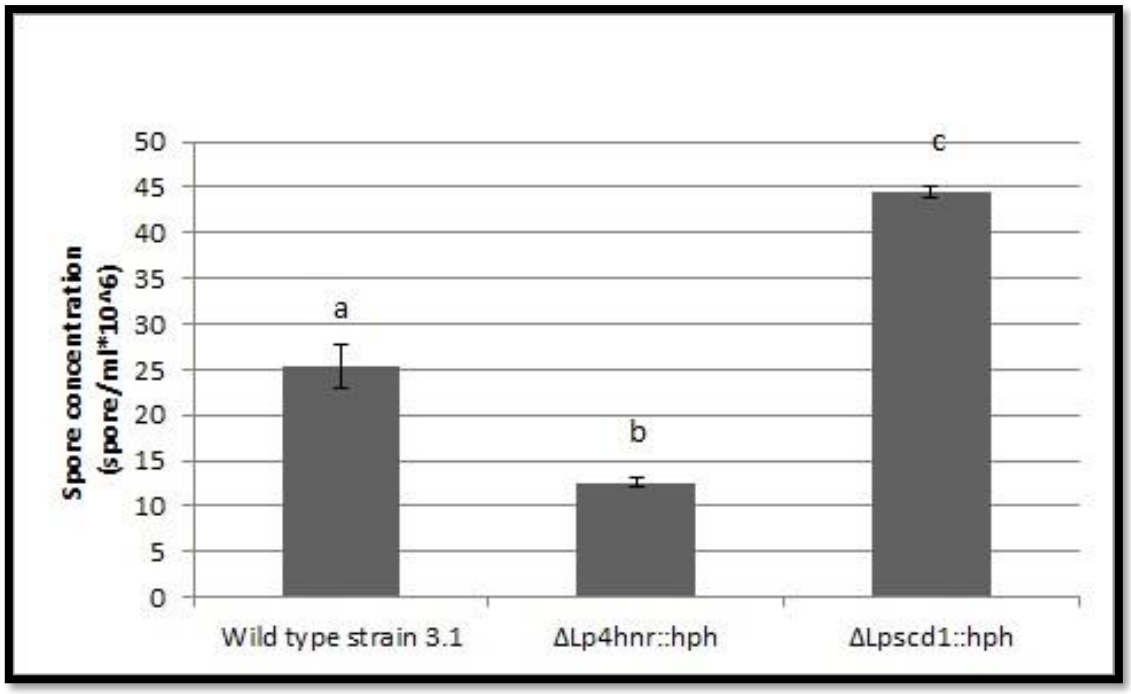

Figure 4.11. Quantification of spore concentrations from 14-day-old OA plate cultures. Spore concentration of the mutant Δ*Lp4hnr*::*hph* was significantly reduced [*P*<0.05, analysis of variance (ANOVA)] when compared to strain 3.1 and mutant Δ*Lpscd1*::*hph*. In contrast, spore concentration was significantly increased in the mutant Δ*Lpscd1*::*hph* when compared to the two other strains. Each bar is the mean of 3 replicates ± standard error and bars with different letters indicate that differences in means are statistically significant at *P*<0.05 (ANOVA).

4.3.5 Sensitivity of Δ*Lpscd1*::*hph* mutant to sulfonylurea

Sensitivity of the mutant Δ*Lpscd1*::*hph* to sulfonylurea was investigated before performing the *SCD1* gene complementation. Figure 4.12 shows growth inhibition of the Δ*Lpscd1*::*hph* mutant on BDCM medium supplemented with 100 µg/ml of sulfonylurea, indicating that the *Magnaporthe oryzae IVL1* sulfonylurea resistance selection system is appropriate for identification of Δ*Lpscd1*::*hph*:SCD complemented transformants.

Figure 4.12. Sensitivity of the Δ*Lpscd1*::*hph* mutant to sulfonyl urea. Lack of growth of strain Δ*Lpscd1*::*hph* on BDCM medium supplemented with 100 µg/ml sulfonyl urea, shows that the mutant is sensitive to the antibiotic and can therefore be used for selection of complemented strains. Scale bar = 1.5 cm.

4.3.6 Complementation of ∆*Lpscd1*::*hph* mutant

Complementation of the mutant ∆*Lpscd1*::*hph* was attempted by random integration of *SCD1* fragments into the mutant genome. The *SCD1* fragment size 3.743-Kb was amplified using primers SD-inf and SD-inr (Table 4.1). By using the IN-Fusion® HD Cloning Kit (Clontech), the *SCD1* fragment was cloned into pCB1532 linearized vector (Section 2.2.2.4). The cloned vector was purified (Section 2.2.2.4.4) and sequenced to confirm correct *SCD1* insertion into the pCBSCD1 vector (Figure 4.13). The vector was then transformed into the ∆*Lpscd1*::*hph* strain and transformants were selected based on resistance to 100 µg/ml sulfonylurea. The complementation procedure was carried out on three separate occasions, but no resistant transformants were recovered.

3410558;Value Read;;Fin;AAA0041154;premix w.temp;final result
;910;">Fin_premix -- 5915 of sequence

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CGAACCGGCGAATCAACATCAACAAGAGCATCAAGAAAATGACAGAGTTGATGCCGACGG

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GCCCGCCCAAGAATTCCTCGCCATGATCTCCAACCCTTCCGTCCTCGGCGATCCCACCCT

CAAAACCCAGCACTTCGTCGGCGCCTCCCGCTGGGAGCGCATCTCCGCCGACGAGGTCGT

CGGCTACCACCAGCTCCGCGTGCCCCATCAGCGCTACGACAGGGACCCGGCACAGCAGGG

CGCCAAGGTCACCGCCAAGGGCCACGCCCACTCCGCCAACAAGCACTGGTACAGAAAGGT

CAATGGCGTGTGGAAGTTTGCCGGTCTGTGTCCTGATATTAGGTGGTCTGAGTATGACTT

TGATAAGATCTTTGCTGGTGGGAGGGAGAACTTTGGGGAGAATGGACAGCAGTGAAGTGG

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GACGTGGACGACGAAAGGGAAGCCAGCCGAGTAGAGCGGTGCAAATTAGCTTATTTTATA

AAACAAGGCGAGAACGGCACTCTGATGAGATTCGGGCTGTGAAATAGGCAGGAGAGGCGC

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GGCCACA";

Figure 4.13. Nucleotide sequences of the *SCD1* fragment cloned into pCB1532 vector for *SCD1* gene complementation of the Δ*Lpscd1*::*hph* mutant. The pCBSCD1 vector was purified and sequenced to confirm successful cloning of *SCD1* fragment (3.743-Kb). The sequencing was performed to identify the five *SCD1* fragments

4.3.7 Restoration of melanin production during mutant interactions

Work conducted here and in Chapter 3 shows that disruption of melanin biosynthetic genes in *L. prolificans* results in altered pigmentation of mutant strains (beige pigmentation of the Δ*Lpscd1*::*hph* mutant, yellow-grey pigmentation of the Δ*Lp4hnr*::*hph* mutant and albinism in the polyketide synthetase-deficient mutant Δ*Lppks1*::*hph*) compared to the grey colouration of the wild type strain 3.1. Plate challenge assays were performed to determine whether hyphal interactions between the albino polyketide synthetase-deficient mutant Δ*Lppks1*::*hph* (Chapter 3) and scytalone dehydratase-deficient mutant Δ*Lpscd1*::*hph* could restore melanin production. Challenge assays show that melanin production (indicated by black pigment production) is restored in the Δ*Lppks1*::*hph* mutant at the point of interaction between the two strains (Figure 4.14).

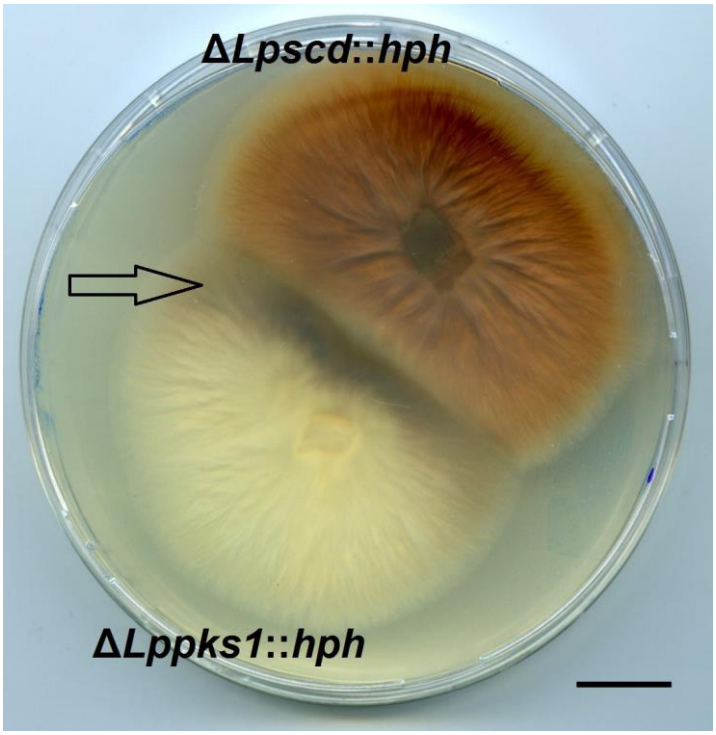

Figure 4.14. Restoration of melanin production during interaction of the mutant strains Δ*Lppks1*::*hph* and Δ*Lpscd1*::*hph*. The mutants were inoculated on either side of a Petri-dish plate containing CM medium and incubated at 30 ^{o}C. At the zone of interaction between the mutants (indicated by the arrow), a black pigment was formed, indicating restoration of melanin production in the Δ*Lppks1*::*hph* mutant. Note also the orange colouration of the Δ*Lpscd1*::*hph* mutant. Scale bar = 1.5 cm.

4.4 Discussion

The aim of this chapter was to disrupt and complement the scytalone dehydratase-encoding gene *SCD1* in *L. prolificans,* to phenotypically characterise the Δ*Lpscd1*::*hph* mutant, and to compare its growth characteristics and pigmentation to the mutant Δ*Lp4hnr*::*hph* developed previously and which is deficient in the melanin biosynthetic enzyme tetrahydroxynaphthalene reductase (4HNR) (Thornton, *et al*., 2015b). The enzyme scytalone dehydratase (SCD) catalyses the reaction scytalone ⇆ 1,3,8-trihydroxynaphthalene (Fig. 4.1), following production of the melanin intermediate scytalone by 4HNR (Bell, *et al*., 1986).

Cloning, sequencing and alignment of the *SCD1* gene confirmed its close homology with scytalone dehydratase-encoding genes in other fungi, while the *L. prolificans* whole genome sequence generated in-house provided sufficient coverage and quality to allow its targeted deletion in the *L. prolificans* wild type strain 3.1 (Thornton, *et al*., 2015b). Targeted deletion of the *L. prolificans SCD1* gene was carried out by replacing the open reading frame of the gene with the gene *hph* (conferring resistance to the antibiotic hygromycin B), following the split marker technique of homologous recombination. This resulted in the successful generation of a Δ*Lpscd1*::*hph* mutant. However, it should be noted that the protoplast transformation process needed to be repeated four times in order to generate a mutant with correct replacement of the *SCD1* ORF with the *hph* transformation cassette. In this study, a large number of ectopic mutants were generated that had an albino phenotype, with Southern blot analysis indicating incorrect integration of the *hph* gene, presumably into other genes involved in melanin biosynthesis. As discussed in the previous chapter, homologous recombination appears to be a rare event in *L. prolificans*, and disruption of the Non-Homologous End Joining (NHEJ) system in this fungus might improve transformation efficiency.

The scytalone dehydratase-deficient mutant Δ*Lpscd*::*hph* developed here showed a dramatic change in pigmentation. While the wild type strain 3.1 is grey in appearance when grown on oatmeal agar, the Δ*Lpscd1*::*hph* mutant is beige. This change in pigmentation is consistent with other fungi in which scytalone dehydratase-encoding genes have been disrupted (Chumley, *et al*., 1990; Tanaka, *et al*., 1991; Wang, *et al*., 2010). Wang and co-workers yielded reddish coloured mutants after disruption of the

corresponding *SCD1* gene in *Grosmannia clavigera* and suggested that this colour was due to the accumulation of the melanin intermediate scytalone (Wang, *et al*., 2010). A similar result was reported in *Sordaria macrospora* where silencing of the scytalone dehydratase-encoding gene *sdh* resulted in reddish coloured mutants (Engh, *et al*., 2007). Similarly, reddish-pink mutants were isolated following disruption of the SCD-encoding gene *arp1* in *Aspergillus fumigatus* (Tsai , *et al*., 1997).

Changes to normal melanin production in fungi can alter their morphology. Tseng and co-workers (Tseng, *et al*., 2014) showed that engineering the entomopathogen *Metarhizium anisopliae* to express DHN-melanin biosynthesis genes increased spore germination, hyphal branching and appressorium production. The Δ*Lpscd*::*hph* mutant generated here showed a significant increase in spore production when compared to the wild-type 3.1, while a significant reduction in spore production was found in the Δ*Lp4hnr::hph* mutant, consistent with a previous study (Thornton, *et al*., 2015b). No significant change in hyphal growth was shown between Δ*Lpscd*::*hph* and strain 3.1 strain, in contrast the mutant Δ*Lp4hnr::hph* mutant, which showed a significant reduction (Thornton, *et al*., 2015b). It is feasible that the decreased sporulation of the Δ*Lp4hnr*::*hph* mutant is directly related to its reduced linear growth rate, with less hyphal biomass capable of producing sporophores. In *Grosmannia clavigera,* SCD-deficient mutants showed no significant differences in sporulation or hyphal growth compared to the wild-type strain (Wang, *et al*., 2010).

When grown on PDA, the Δ*Lpscd1*::*hph* mutant produced an orange discolouration, suggesting diffusion of the melanin intermediate scytalone into the medium. Since the Δ*Lpscd1*::*hph* mutant lacks the enzyme scytalone dehydratase, it is unable to convert scytalone (the product of the enzyme 4HNR) to 1,3,8-trihydroxynaphthalene (1,3,8-THN) and so scytalone is accumulated (Fig. 4.1). To test this hypothesis, the Δ*Lpscd1*::*hph* mutant was grown in dual culture with the albino polyketide synthetase-deficient mutant Δ*Lppks1*::*hph* developed in Chapter 3. Conversion of soluble scytalone produced by the Δ*Lpscd1*::*hph* mutant by functional SCD present in the Δ*Lppks1*::*hph* mutant would result in the production of melanin, demonstrated by a black colour formation in the zone of hyphal interaction. Indeed, the Δ*Lppks1*::*hph* turned black in the zone of interaction indicating the restoration of melanin production in this mutant. This result is consistent with the observations of Wang and co-workers

(Wang, *et al*., 2010) who showed that a polyketide synthetase-deficient mutant of *Grosmannia clavigera* turned black during hyphal interaction plate with a scytalone dehydratase-deficient mutant of the same fungus.

Restoration of melanin production would also be expected in the Δ*Lpscd1*::*hph* mutant following complementation of enzyme-deficient strain. A previous study was successful in restoring SCD activity and pigmentation (Kubo, *et al*., 1996), but despite repeated attempts to transform the *L. prolificans* mutant with an *SCD1* fragment comprising the ORF and sequences upstream and downstream of the ORF (as promoter and terminator respectively), complementation was not possible. The likely reason for this is the poor quality of the promoter and terminator region sequences of the *SCD1* gene identified in the in-house *L. prolificans* 3.1 whole genome sequence (results not shown), which meant that the size of these regions could only be determined speculatively.

Despite this, work conducted in this chapter resulted in the successful generation of a mutant strain deficient in the enzyme SCD. Successful generation of the Δ*Lpscd1*::*hph* mutant now enables comparative studies to be made of mutant survival to environmental stresses and resistance to killing by cells of the mammalian innate immune system. The results of these studies are reported in Chapters 5 and 6.

5. Result

Protective role of DHN-melanin against environmental stress in *L. prolificans*

5.1 Introduction

5.1.1 The survival advantages of melanin in fungi exposed to harsh environmental conditions

Fungi are exposed to various hostile environmental conditions, such as extreme temperature, pressure, and/or radiation (Brown, *et al*., 2014). Living organisms have developed various mechanisms to survive such extreme conditions (Brown, *et al*., 2014). The biosynthesis of melanin in fungi is associated with survival advantages and enables living organisms to resist extreme conditions. However, melanin is not essential for fungal growth (Fogarty, *et al*., 1996). In some circumstances, melanin biosynthesis is triggered in response to certain stresses, such as DNA damage by UVR-induced pigmentation for example when the wild-type strain of *Gaeumannomyces graminis* var. *graminis* is exposed to UVR (Frederick, *et al*., 1999; Henson, *et al*., 1999).

Melanin is regarded as photoprotective due to its ability to absorb ultraviolet radiation (UVR). Melanin has therefore found commercial use as sunscreen (Hill, 1992 ; Herrling , *et al*., 2008). Sun is a natural source of UVR, which is separated into three categories according to wavelength: UVA, UVB and UVC (Brenner & Hearing, 2008). UVB has a short wavelength – about 280-320 nm – and is more harmful and cytotoxic to living organisms than UVA (Brenner, *et al*., 2008). The main reason UVB is so harmful is due to the fact that it is absorbed directly by DNA, as oppose to the indirect effect of UVA, which generates reactive oxygen species (ROS) (Brenner & Hearing, 2008). The protective role of melanin in *Cryptococcus neoformans* was demonstrated when a non-melanised strain was shown to be more sensitive to UVR than a melanised strain (Wang & Casadevall, 1994). Melanised pycnidia protect pycnidiospores from UV light, which was proven when melanised pycnidia and an albino mutant were exposed to UVR in *Ascochyta rabiei* (Akamatsu, *et al*., 2010).

In vitro studies have shown that DHN-melanin biosynthesis in *Gaeumannomyces graminis* var. *graminis* is induced when the fungi are grown in a copper-supplemented medium (Caesar-Tonthat, *et al*., 1995). It was found that accumulation of melanin in the mycobiota is high in extreme conditions, for instance melanised spores dominate in the aerial environment more than in the soil (Gessler, *et al*., 2014). Melanin quantity affects the degree of protection. It was demonstrated that *Aspergillus niger* has adapted to produce higher quantities of melanin in regions exposed to higher solar radiation (Singaravelan, *et al*., 2008).

Moreover, melanin protects fungi against ionising radiation (Dadachova & Casadevall, 2009). The uptake capacity of radioisotopes radiocobalt (Co-60) and radiocesium (Cs-137) by melanised fungi *Alternaria alternate* is greater than in the non-melanised *Aspergillus pulverulens* or *Fusarium verticilloides* (Mahmoud, 2004). Radionuclide contamination controls fungal community composition in the contaminated area, encouraging a dominance of melanised fungi in the mycobiota (Dighton, *et al*., 2008). The existence of melanised fungi in the radioactively contaminated soils at the Nevada Test Site and around the damaged Chernobyl nuclear reactors highlights the ability of melanised fungi to absorb and clarify radioactive contamination (Eisenman & Casadevall, 2012).

On the other hand, melanin has been shown to be a contributing factor in protecting against extreme temperatures (hot and cold) in *Cryptococcus neoformans* and *Wangiella dermatitidis* (Rosas & Casadevall, 1997; Jr, *et al*., 2006). Pigments play a thermoprotective role, which is why melanised fungi inhabit extreme regions such as the Antarctic and Arctic. However, fungi have developed numerous mechanisms to resist extreme temperatures (Robinson, 2001). Melanin enhances fungal survival in high temperatures and desiccation in the plant pathogen *Monilinia fructicola* (Rehnstrom, *et al*., 1996). This resistance created by melanin enhances cell wall integrity (Rehnstrom, *et al*., 1996). Moreover, melanised and human pathogens *Exophiala dermatitidis* and *Exophiala phaeomuriformis* are able to survive in dishwashers, tolerating high temperatures and detergents (Zalara, *et al*., 2011).

Resistance to lytic enzymes is an additional feature that melanin bestows upon the melanised fungi (Bloomfield, *et al*., 1967). It was noticed that melanin-deficient mutants were more susceptible to lytic enzymes in comparison to the wild-type strain

Aspergillus nidulans (Kuo , *et al*., 1967). This could be due to enzyme confines on melanin owing to melanin cross-link polysaccharide in the cell wall constitution or to steric hindrance (Jacobson, 2000).

Melanin possesses binding sites to metal, such as carboxyl and phenolic groups (Fogarty, *et al*., 1996). It was shown that melanin has an affinity to a range of metal ions, including copper, calcium and magnesium (Larsson, *et al*., 1978). The protective role of melanin against silver nitrate ($AgNO_3$), a material toxic to fungi, was studied in *Cryptococcus neoformans* strains; results pointed to an increased susceptibility in non-melanised strains compared to melanised strains (García-Rivera, *et al*., 2001). Adsorption of copper was significantly higher in melanised *Aureobasidium pullulans* and *Cladosporium resinae* than in albino strains, while purified melanin adsorbed copper better than melanised cells. A low pH also enhances copper-binding efficiency (Gadd, *et al*., 1988).

Purified melanin from *Hortaea werneckii* has antimicrobial properties against *Salmonella typhi, Vibrio parahaemolyticus* and *Klebsiella pneumonia* (Rani, *et al*., 2013).

Melanin protects *Cryptococcus neoformans* from the soil predator *Acanthamoeba castellanii*, with studies having shown that the melanised strain survives after ingestion by *Acanthamoeba castellanii* amoeba (Steenbergen, *et al*., 2001). Another study investigated the role of melanin in shielding *Aspergillus fumigatus* from phagocytosis by *Dicytostelium discoideum,* which is a soil predator amoeba. It was shown that non-melanised conidia phagocytized more efficiently than melanised conidia (Hillmann , *et al*., 2015).

Melanin enables melanised species *Hortaea werneckii, Phaeotheca triangularis, Trimmatostroma salinum, Aureobasidium pullulans* and *Cladosporium* spp. to survive in the hypersaline waters of salterns at a concentration of 3-30% NaCl (Gunde-Cimerman, *et al*., 2000). Kogej *et al*., (2007) found that melanin retains a high concentration of glycerol in the cells of *Hortaea werneckii* to reduce the entrance of salt as an osmoadaptation (Kogej, *et al*., 2007).

Melanised fungi such as *Alternaria alternata, Cladosporium cladosporioides,* and *Urocladium botrytis* resist technological contamination from different polluters such as heavy metals, and are prevalent in urban roadside areas (Kul'ko & Marfenina, 2001).

The dominant presence of the black endophytic fungi species, which was isolated from *Deschampsia antarctica Desv. (Poaceae)* leaves, confirmed the contribution of melanin to survival advantages in hostile Antarctic conditions (Rosa, *et al*., 2009).

Melanin is also involved in the reduction of fungal sensitivity to the antifungal drugs amphotericin B and caspofungin, this observation was recorded in *Cryptococcus neoformans* and *Histoplasma capsulatum,* when non-melanised strains showed greater sensitivity in comparison to the melanised strain following a killing assay (van Duin, *et al*., 2002). Consistent with this result, the polyketide synthase deficient mutant of *Wangiella dermatitidis* showed greater sensitivity to antifungal drugs than the melanised parent strain (Jr, *et al*., 2006).

Melanin's ability to bind antifungal drugs has been investigated, with the result that the incubation of melanin with amphotericin B and caspofungin alters the composition of the melanin C:N ratio (van Duin, *et al*., 2002).

It has been demonstrated that melanin is a redox buffer; free electrons enable melanin pigments to interact with oxidants, thereby raising fungal tolerance to oxidants (Jacobson, 2000). Jacobson *et al*. (1995) used melanised and non-melanised strains to assess the protective role of melanin against oxidants. The results demonstrated that melanin protects fungi from oxidants such as permanganate and hypochlorite (Jacobson, *et al*., 1995). Incubation of melanised and non-melanised *Cryptococcus neoformans* with nitrogen and oxygen-derived oxidants showed a higher survival rate of melanised fungi compared to non-melanised (Wang, *et al*., 1994a). The melanin isolated from *Hypoxylon archeri* showed quenching activity against free radicals, HOCl or H_2O_2, by following an oxidant-competing system between melanin and oxidation of 5-thio-2-nitrobenzoic acid (TNB), which is oxidised by free radicals (Wu, *et al*., 2008). Melanin demonstrated a greater ability to eliminate free radicals (Wu, *et al*., 2008). Melanin-deficient mutants of *Penicillium marneffei* are sensitive to killing by H_2O_2 (Woo, et al., 2010). Tam *et al*. (2015) reviewed other non-melanised mutants sensitive to killing by hydrogen peroxide such as *Paracoccidioides brasiliensis, Aspergillus fumigatus* and *Sporothrix schenckii* (Tam, *et al*., 2015).

5.1.2 The aim of this chapter

The aim of this chapter was to investigate the protective role that melanin plays against environmental stress in *L. prolificans*. To do this, the survival percentage of melanised wild-type strain 3.1 of *L. prolificans* and non-melanised strains generated in chapters 3 and 4, Δ*Lppks1*::*hph,* Δ*Lpscd*::*hph*, Δ*Lp4hnr*::*hph* (Thornton, *et al*., 2015b)**,** and the complemented strain Δ*Lppks1*::*hph*:*PKS* of Δ*Lppks1*::*hph* mutant (chapter 3)*,* was calculated after exposure to different environmental conditions.

It was hypothesised that melanised strains would show greater resistance to harsh environmental conditions than non-melanised strains.

I set out to investigate the protection role that melanin plays to protect fungi from UV radiation, extreme temperature (hot and cold), oxidative killing by hydrogen peroxide H_2O_2, and antifungal drugs including polyene amphotericin B (AmB), the azoles voriconazole (Vor) and posaconazole (Pos), and the echinocandins caspofungin (Casp) and anidulafungin (Anid).

5.2 Material and methods

5.2.1 Lyophilisation of fungal mycelium

A 14 days old oatmeal agar plate (OA) of *L. prolificans* was flooded with 20 ml MQ, agitated with an L-shaped spreader and the suspension was filtered through Miracloth to separate spores from mycelium. The spore suspension was transferred to 1.5 ml micro-centrifuge tube and spores were recovered by centrifugation at 14 000 x g for 5 min. The spores were washed three times with MQ and centrifuged at 14,000 x g for 5 min. The spore concentration was adjusted to 10^6 spore/ml. One hundred ml of SDB medium were inoculated with 200 µl of spore suspension and incubated with shaking (75 rpm) in an Innova 4000 rotary incubator (New Brunswick Scientific) at 30 °C for 48 h. The mycelium was collected by Miracloth, washed and kept in liquid N_2 until lyophilized (Heto Power dry LL3000 Freeze dry, Thermo Fisher Scientific). The lyophilized mycelium was stored at 4 °C until required.

5.2.1.1 Enzyme-linked immunosorbent assay (ELISA)

An ELISA was performed using *L. prolificans*-specific monoclonal antibody CA4 that binds to the melanin biosynthesis enzyme tetrahydroxynaphthalene reductase (Thornton & Wills, 2015 b). One mg of lyophilized mycelium was suspended in 1 ml phosphate buffered saline (PBS: 0.8% NaCl; 0.02% KCl; 0.115% Na_2HPO_4; 0.02% KH_2PO_4; pH7.2) to extract antigen, centrifuged for 5 min at 14,000 x g and then the wells of a microtitre were coated with 50 µl of the supernatant containing solubilized antigens. The plate was sealed in a plastic bag and incubated overnight at 4 °C. CA4 hybridoma was added to the wells, which contained immobilized antigen, incubated for 1 h, followed with addition of goat anti-mouse polyvalent (immunoglobulin classes IgG, IgA, and IgM). Peroxidase conjugate (A-0412; Sigma Chemical Company, Poole, United Kingdom) was added after a 1:1000 dilution in PBST (phosphate-buffered saline containing 0.05% (v/v) Tween-20) and the plate was incubated for 1 h at room temperature. 50 µl of tetramethylbenzidine (TMB) (T-2885; Sigma) substrate solution was added and incubated for 30 min. The reactions were stopped by adding 3 M H_2SO_4. An MRX automated microplate reader (Dynex Technologies, Billingshurst, UK) was used to determine the absorption value at 450 nm. Wells were washed for 5 min with PBST between incubations. TCM (tissue culture medium) containing 10% (v/v) fetal bovine serum was used as a control.

5.2.2 Sensitivity to UV Radiation

Preliminary investigations aimed to establish the UV dose at 254 nm that resulted in a 50% reduction in survival of the wild-type strain 3.1. Replicate spore suspensions with a concentration of 10^3 spores/mL were exposed to UV light generated by an HL-2000 HybriLinker Hybridization oven (UVP) with doses of 100, 200, 300, 400 and 500 mJ/cm^2. Following exposure, 300 µL of spore suspensions were spread on the surface of SDA plates and incubated for 3 days at 30 ˚C in the dark. The control consisted of non-irradiated spores and percentage survival was determined from the numbers of colonies that developed from irradiated spores compared to the non-irradiated control. Through this process it was determined that a UV dose of 200 J/cm^2 resulted in 50% survival of irradiated spores of the wild-type compared to the matched non-irradiated control. All subsequent experiments using mutant strains were conducted at this dose and percentage survivals relative to matched controls determined as described. There were 3 replicates for each strain and experiments were conducted three times.

5.2.3 Sensitivity to extreme temperature (heating and freezing)

Preliminary investigations assessed the temperature parameters that reduced fungal survival of the wild-type strain 3.1. Two-week-old spores were harvested from the wild-type strain 3.1, washed three times with MQ and adjusted to a concentration of 10^3 spores/ml in an Eppendorf tube. Spore suspensions were exposed to a range of temperatures (42 °C, 45 °C, and 49 °C) in a pre-heated block heater (Stuart block heater; SBH130D) for one hour, then plated on SDA plates and incubated for three days at 30 °C. The temperature degrees 49 °C, 50 °C, 51 °C, 53 °C and 55 °C were selected to compare the difference in survival percentage between melanised and non-melanised strains of *L. prolificans*. Three replicates of each *L. prolificans* strain were exposed to 49 °C, 50 °C, 51 °C, 53 °C and 55 °C for one hour, plated on SDA plates and incubated for three days at 30 °C.

Three replicates of spore suspensions at a concentration of 10^3 spores/ml were frozen in the freezer at -20 °C for one, three, five and seven days, then thawed at room temperature. After freezing, 300 µl of the spore suspension was spread onto SDA plates and incubated at 30 °C for three days. Untreated (neither heated nor frozen) spore suspensions were spread onto SDA plates on the same day of the experiment as a control to prevent change in viability. Survival percentages were calculated by

counting the number of colonies derived from germinated spores relative to the controls. Experiments were repeated three times.

5.2.4 Sensitivities to hydrogen peroxide (H_2O_2)

Preliminary investigations set out to establish the molarity and exposure time to H_2O_2 that resulted in a 50% reduction in survival of the wild-type strain 3.1. Replicate spore suspensions with a concentration of 10^3 spores/mL were suspended in 100 mM phosphate buffer (pH 7.0) comprising a range of mM concentrations of H_2O_2. Following exposure, 300 µL of spore suspensions were spread on the surface of SDA plates and incubated for 3 day at 30 °C in the dark. The control consisted of untreated spores (suspended in buffer only) and percentage survival was determined from the numbers of colonies that developed from treated spores compared to the untreated control. Through this process it was determined that exposure of spores to 160 mM H_2O_2 for 60 min resulted in 50% survival of treated spores of the wild-type compared to the matched untreated control. All subsequent experiments using mutant strains were conducted using this same treatment regime and percentage survival relative to matched controls determined as described. There were 3 replicates for each strain and experiments were conducted three times.

5.2.5 Sensitivity to antifungal drugs

The sensitivity of *L. prolificans* strains to antifungal drugs were investigated following two methods:

5.2.5.1 Epsilometer test (E-test) assay

E-test assay were used to investigate the sensitivity of *L. prolificans* strains to antifungal drugs following the manufacturer's instructions (BioMerieux). Five antifungal agents were used including the polyene amphotericin B (AmB), the azoles voriconazole (Vor) and posaconazole (Pos), and the echinocandins caspofungin (Casp) and anidulafungin (Anid). 200 µl of spore suspensions 10^5 spores/ml were spread on Sabouraud dextrose agar (SDA) plates, the E-test strips were applied centrally and incubated at 30 °C for 48 to 72 h. The MIC values were read after 24 h and 48 h after incubation. Plates were examined daily. Three individual experiments were repeated.

5.2.5.2 Sensitivity to amphotericin B by using dry weight assay

The sensitivities of the mutants and strain 3.1 to the polyene amphotericin B (AmB; Sigma, A2942) were tested using a liquid culture method. Tissue culture medium was amended with a liquid formulation of AmB (Sigma; A2942) to give a final concentration of 32 µg/mL and the control consisted of TCM amended with an equivalent volume of sterile MQ-H_2O only. Tissue culture flasks (75 cm^2) containing AmB or control media were inoculated with spores of the *L. prolificans* strains, or the drug-sensitive pathogen *Aspergillus fumigatus* (strain AF293), to give a final concentration of 10^6 spores/mL. Flasks were incubated for 3 days at 30 ˚C with shaking (48 rpm) in an Innova 4000 rotary incubator and the mycelium was harvested, dried at 70 ◦C for 48 h and dry weights obtained. The experiment was repeated three times with three replicates for each strain and treatment.

5.3 Results

5.3.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Lyophilised mycelium of *L. prolificans* strains was used to extract protein for an ELISA. The ELISA was carried out by using the *L. prolificans*-specific monoclonal antibody CA4, which targets the melanin biosynthesis enzyme tetrahydroxynaphthalene reductase (Thornton, et al., 2015 b). All strains interacted positively with mAb CA4, with the exception of the Δ*Lp4hnr*::*hph* mutant (Figure 5.1). Results confirmed that the gene disruption targeted a specific gene.

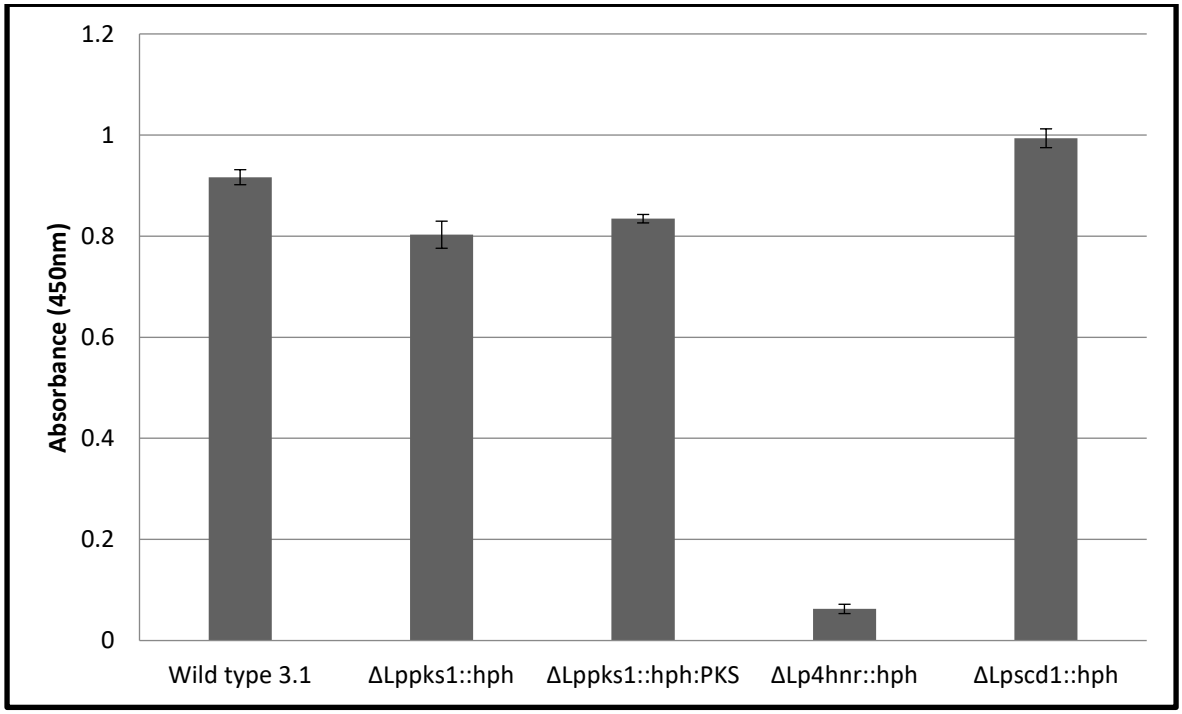

Figure 5.1. ELISA absorbance value at 450 nm of tetrahydroxynaphthalene reductase (antigen) extracted from lyophilised mycelium of *L. prolificans* strains. The *L. prolificans*-specific monoclonal antibody CA4 interacts positively with all strains, with the exception of the Δ*Lp4hnr*::*hph* mutant.

5.3.2 Sensitivity to UVR

Relative to the non-exposed control sample, 51% of the wild-type strain 3.1 samples survived exposure to 200 J/cm^2 of UVR. Under the same conditions, results showed a significant decrease in the survival rate of the Δ*Lppks1*::*hph* mutant (about 28%) in comparison to the wild-type strain 3.1. No difference was observed between the Δ*Lpscd1*::*hph* and Δ*Lp4hnr*:: *hph* strains, where the survival percentage stood at about 40% and 42% respectively compared to the wild-type strain 3.1. The complemented strain of the Δ*Lppks1*::*hph* mutant restored melanin's protective role, producing a survival percentage of about 63% and thus showing no significant difference to the wild-type strain 3.1 (Figure 5.2). Figure 5.3 shows the growth of *L. prolificans* colonies derived from germinated spores after the spore suspension was exposed to 200 J/cm^2 of UVR, spread onto SDA plates and incubated in the dark at 30 ˚C for three days.

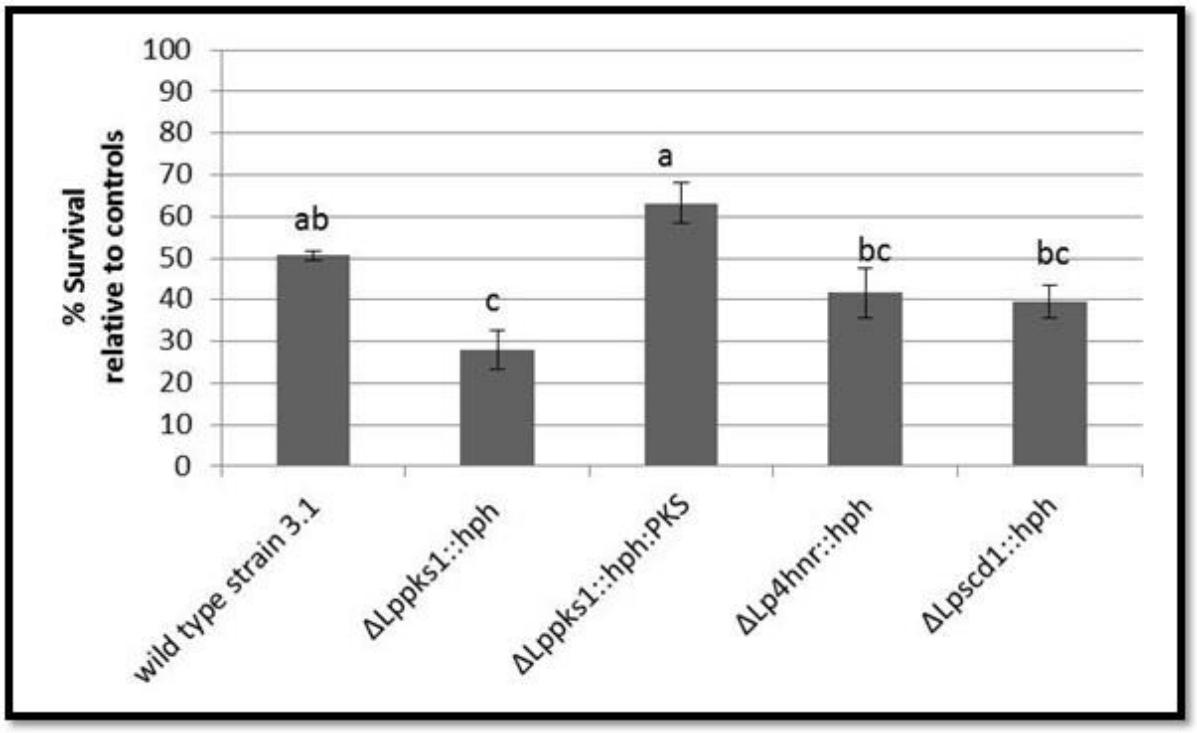

Figure 5.2. Sensitivity of *L. prolificans* strains to UVR. Spore suspensions at a concentration of 10^3 spores/ml of *L. prolificans* strains were exposed to 200 J/cm^2, spread onto SDA plates and then incubated for three days at 30 °C in the dark. The survival percentage was calculated by counting the number of colonies derived from germinated spores and was determined relative to the control. The Δ*Lppks1*::*hph* mutant showed a significant reduction in survival percentage compared with the wild-type strain 3.1, while other strains showed no significant differences. Similarly, the Δ*Lppks1*::*hph*:*PKS* strains restored their resistance to UVR. Each bar represents the mean of three replicate samples ± SE. Bars sharing similar letters indicate that differences in means are not statistically significant at $P<0.05$ (ANOVA).

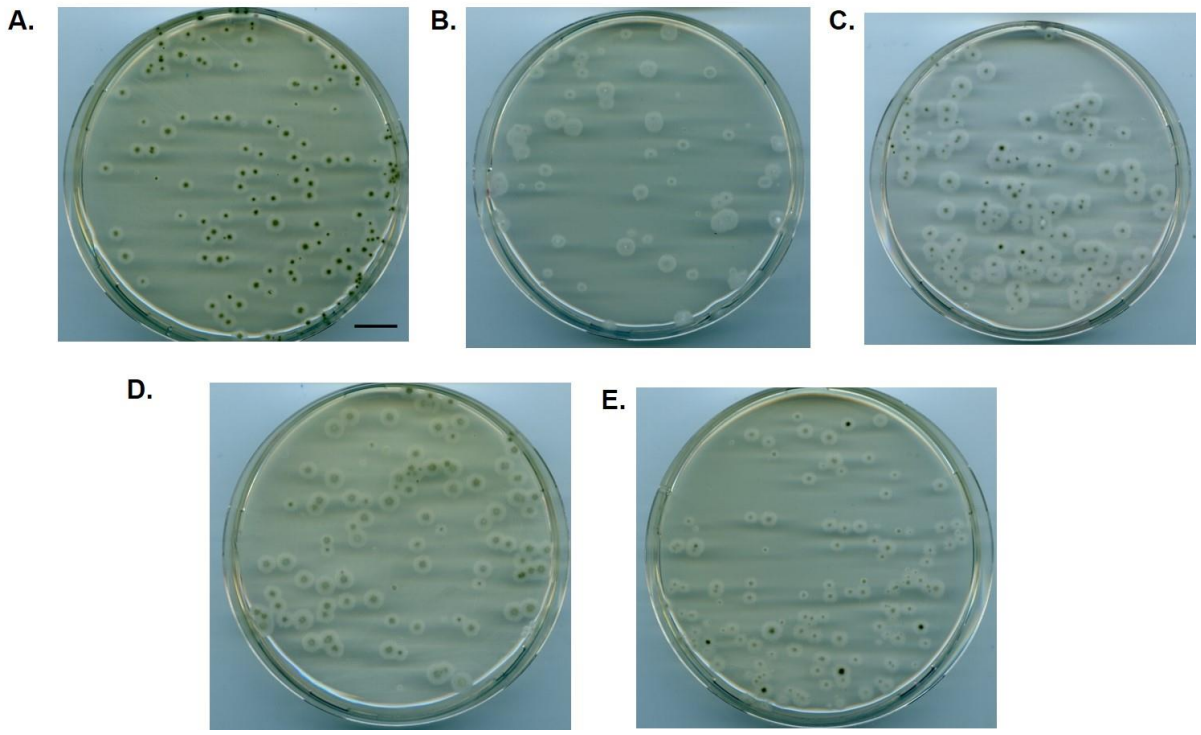

Figure 5.3 Growth of irradiated spores of *L. prolificans* strains. 300 µl of irradiated spore suspension (10^3 spores/ml) of each *L. prolificans* strain was separated into SDA plates and incubated for three days at 30 °C in the dark. The dose of irradiation was 200 J/cm^2 UVR (254 nm). **A)** wild-type strain 3.1 **B)** *ΔLppks1::hph* mutant **C)** *ΔLppks1::hph:PKS* complemented strain **D)** Δ*Lp4hnr*::*hph* mutant **E)** *ΔLpscd::hph* mutant. Images were captured using an Epson Expressi on 1680 Pro scanner. Scale bar that applied to all images=1 cm.

5.3.3 Sensitivity to extreme temperature (heating and freezing)

The sensitivity of melanised and non-melanised strains of the *L. prolificans* to extreme temperatures was assessed. 10^3 spores/ml of *L. prolificans* strains were heated at various temperatures for one hour, spread onto SDA plates and incubated for three days at 30 °C. Spore suspensions exposed to temperatures lower than 49 °C showed no killing, while temperatures higher than 53 °C and 55 °C resulted in the killing of most spores. The survival percentage was determined by the number of colonies derived from germinated spores relative to the control (Figure 5.5). The results suggest that there is no significant difference between the melanised wild-type strain 3.1 and the non-melanised mutants in terms of survival percentage in this temperature range (Figure 5.4).

Similarly, 10^3 spores/ml of *L. prolificans* strains were frozen at -20 °C for one, three, five and seven days, then thawed at room temperature, spread onto SDA plates and incubated for three days at 30 °C. The survival percentage was determined by the number of colonies derived from germinated spores relative to the control (Figure 5.7) No significant differences in survival percentage were found between melanised and non-melanised strains (Figure 5.6). These results indicate that melanin plays no role in the survival of *L. prolificans* in extreme temperatures.

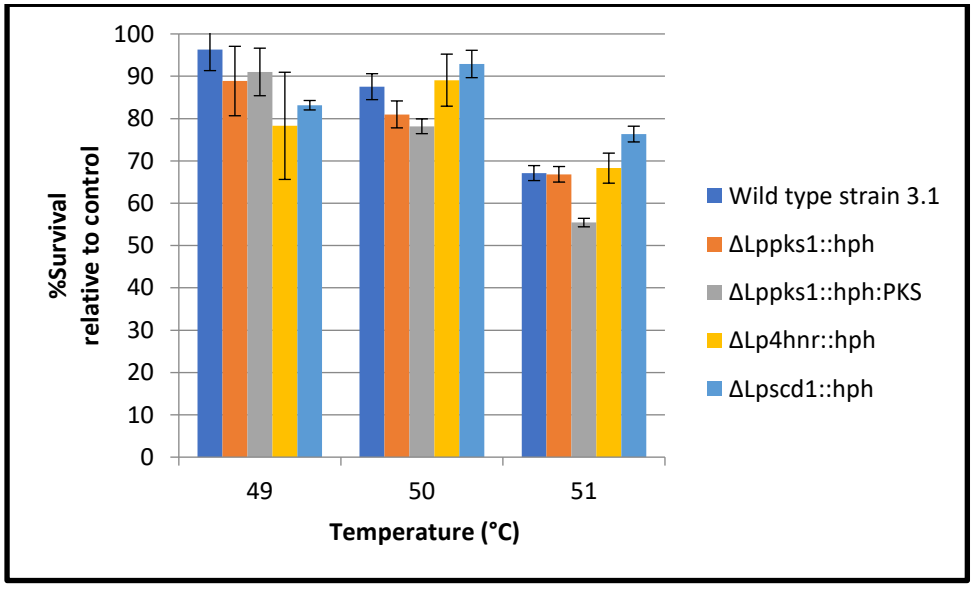

Figure 5.4. Sensitivity of *L. prolificans* strains to high temperatures. 300 µl of spore suspension (10^3 spores/ml) of *L. prolificans* strains was exposed to temperatures ranging from 49-51 °C for one hour. No significant difference was observed in survival percentage between melanised and non-melanised strains. These results reveal that melanin plays no protective role in *L. prolificans* against high temperatures. Each bar represents the average of three replicate plates ± SE ($P > 0.05$).

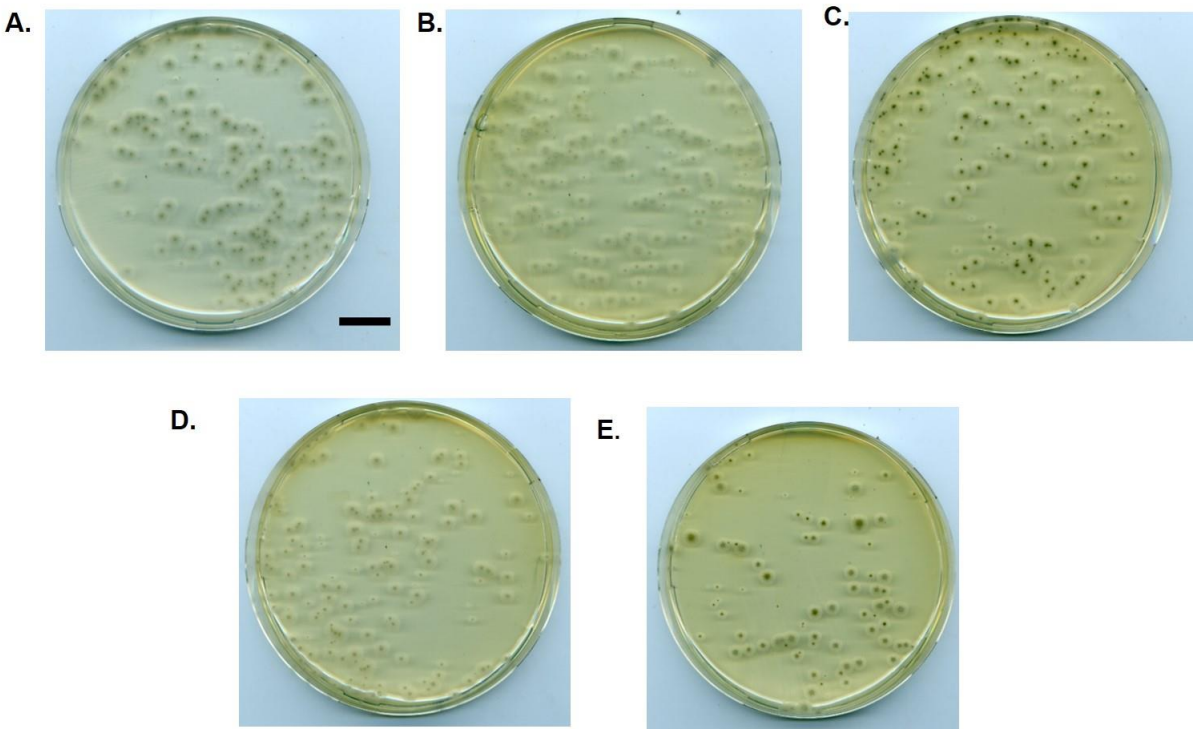

Figure 5.5. Growth of *L. prolificans* spores after being heated for 60 min. at 51°C. Spore suspensions were heated for 60 min., 300 µl of aliquot was spread onto SDA plates and then incubated at 30 °C for three days. **A)** wild-type strain 3.1 **B)** *ΔLppks1::hph* mutant **C)** *ΔLppks1::hph:PKS* complemented strain **D)** Δ*Lp4hnr*::*hph* **E)** *ΔLpscd::hph.* Images were captured using an Epson Expressi on 1680 Pro scanner. Scale bar that applied to all images=1.5 cm.

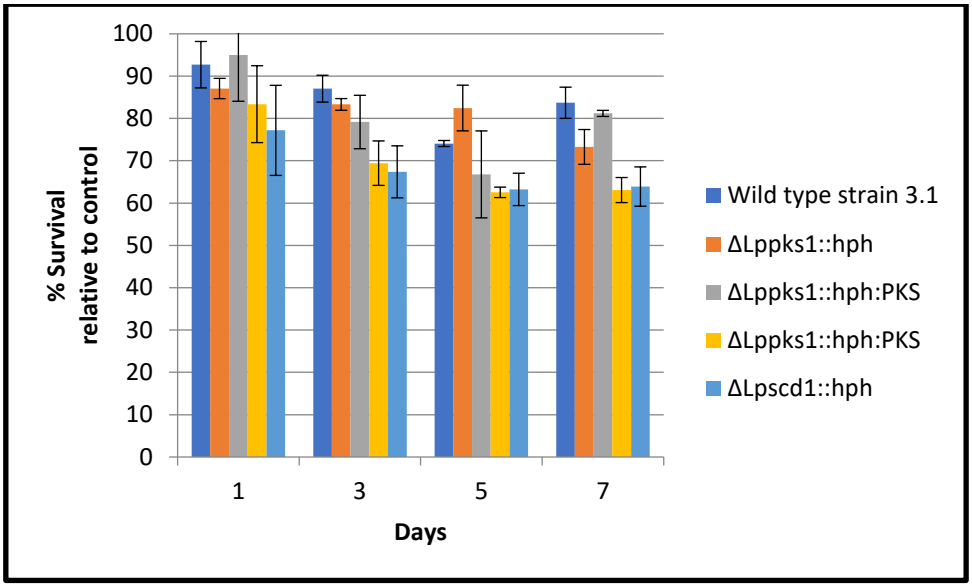

Figure 5.6. Sensitivity of *L. prolificans* strains to freezing. Spore suspensions of the *L. prolificans* strains were frozen at -20 °C for one, three, five or seven days, thawed at room temperature, spread onto SDA plates and incubated for three days at 30 °C. Results showed no significant difference between the survival percentage of melanised and non-melanised strains after freezing. Each bar represents the average of three replicate plates ± SE ($P > 0.05$).

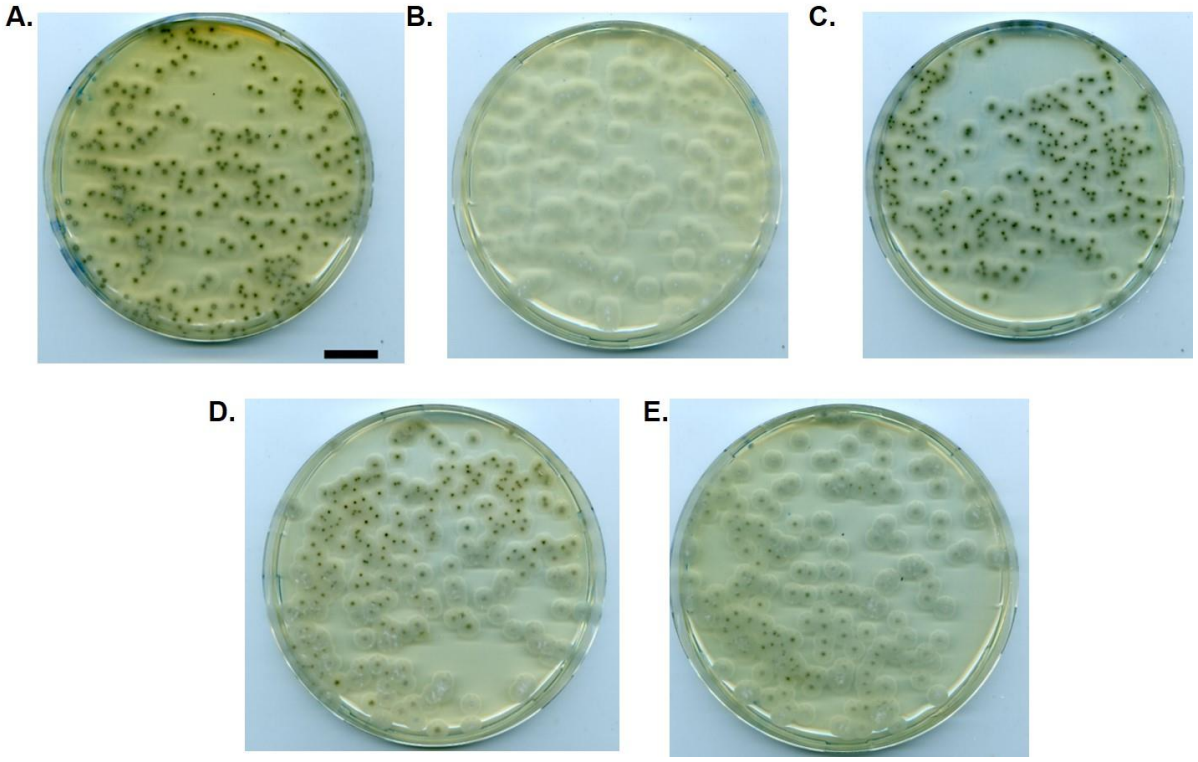

Figure 5.7. Growth of *L. prolificans* spores after freezing. Spore suspensions of *L. prolificans* strains were frozen for three days at -20 °C, thawed at room temperature, spread onto SDA plates and then incubated for three days at 30 °C. **A)** wild-type strain 3.1 **B)** *ΔLppks1::hph* mutant **C)** *ΔLppks1::hph:PKS* complemented strain **D)** Δ*Lp4hnr*::*hph* **E)** *ΔLpscd::hph*. Images were captured using an Epson Expressi on 1680 Pro scanner. Scale bar that applied to all images=1.5 cm.

5.3.4 Sensitivity to hydrogen peroxide (H_2O_2)

It was hypothesised that melanin protects fungi from oxidative killing by H_2O_2 owing to its antioxidant properties and its ability to scavenge damaging reactive oxygen species (ROS) (Jacobson, 2000; Goncalves, et al., 2005). The survival percentage of the wild-type strain 3.1 was about 50% after exposure to 160 mM for one hour. Following this, all *L*. *prolificans* strains were exposed to the same conditions to investigate the protective role of melanin in *L. prolificans*. The results showed a significant reduction in the survival percentages of Δ*Lppks1*::*hph* and Δ*Lp4hnr*:: *hph* strains compared to the wild type (21% and 24% respectively relative to the control). No significant difference in survival percentage was observed between the Δ*Lpscd1*::*hph* strain and the wild-type strain 3.1. The complemented strain (Δ*Lppks1*::*hph*:*PKS*) showed no significant difference in survival percentage, indicating that melanin restores the protective role of pigmentation against H_2O_2 (Figure 5.8). Figure 5.9 shows the growth of *L. prolificans* strains in SDA plates after exposure to 160 mM of H_2O_2 for one hour.

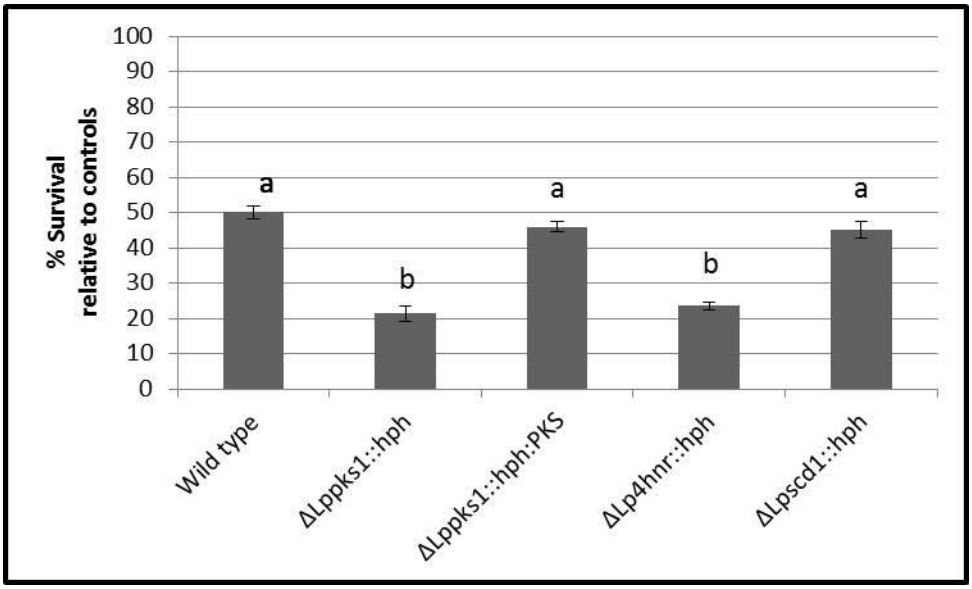

Figure 5.8: Sensitivity of *L. prolificans* strains to hydrogen peroxide (H_2O_2). 10^3 spores/ml of *L. prolificans* strains were exposed to 160 mM for 60 min., spread onto SDA plates and incubated for three days at 30 °C in the dark. Results showed a significant reduction in survival percentage among the Δ*Lppks1*::*hph* and Δ*Lp4hnr*:: *hph* strains, while no significant difference was observed between the Δ*Lpscd1*::*hph* strain and the wild-type strain 3.1. Similarly, the Δ*Lppks1*::*hph*:*PKS* complemented strain of Δ*Lppks1*::*hph* restored the protective role of pigmentation as results showed no significant difference in survival percentage compared with the wild-type. The survival percentage was determined by the number of colonies derived from germinated spores relative to the control sample. Each bar represents the mean of three replicate samples ± SE. The different letters a, b and c show a significant difference ($P< 0.05$).

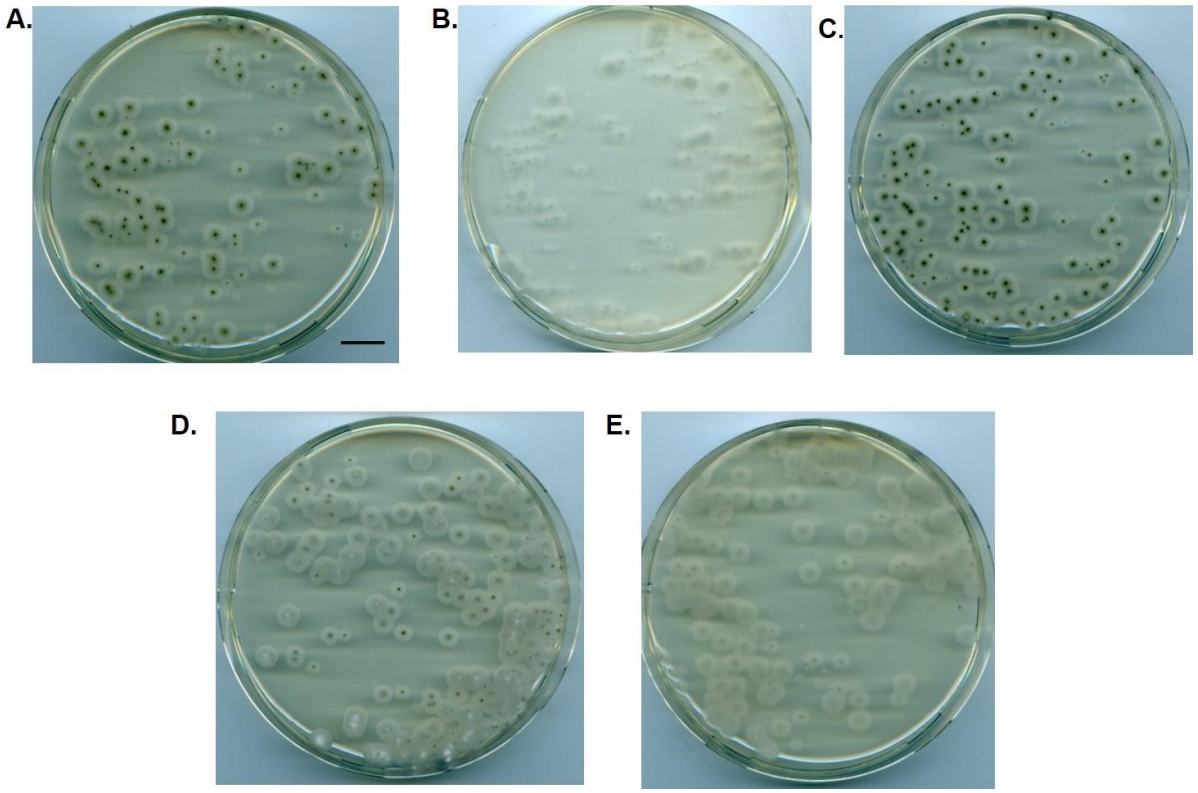

Figure 5.9: Growth of *L. prolificans* strains treated with H_2O_2. Growth of *L. prolificans* strains treated with 160 Mm of H_2O_2 for one hour, separated between SDA plates and incubated at 30 °C for three days. **A)** wild-type strain 3.1 **B)** *ΔLppks1::hph* mutant **C)** *ΔLppks1::hph:PKS* complemented strain **D)** Δ*Lp4hnr*::*hph* mutant **E)** *ΔLpscd::hph* mutant. Images were captured using an Epson Expressi on 1680 Pro scanner. Scale bar that applied to all images=1 cm.

5.3.5 Sensitivity of *L. prolificans* strains to antifungal drugs

L. prolificans resists all antifungal drugs (Hopwood, *et al*., 1995). It was hypothesised that melanin confers resistance to antifungal drugs. The recent study set out to investigate the protective role that melanin plays against antifungal drugs by following two different methods.

5.3.5.1 Epsilometer test (E-test) assay

The protective role of melanin against the five antifungal drugs – the polyene amphotericin B (AmB), the azoles voriconazole (Vor) and posaconazole (Pos), and the echinocandins caspofungin (Casp) and anidulafungin (Anid) – was examined using an E-test assay. E-test strips containing a gradual concentration of the antifungal drug were placed in the middle of SDA plates, covered with 200 µl of 10^5 spores/ml and incubated at 30 °C for three days. The maximum concentration of drug on each strip was 32 µg/ml. Control plates containing no E-test strips were covered with 200 µl of spore suspension. After 48 hours, the fungi had grown to cover the plate without any inhibition zones around the strips, thus indicating that all the strains are resistant to these five antifungal drugs with MIC ≥32 µg/m (Figure 5.10). This result showed that melanin has no effect on the resistance of *L. prolificans* to antifungal drugs.

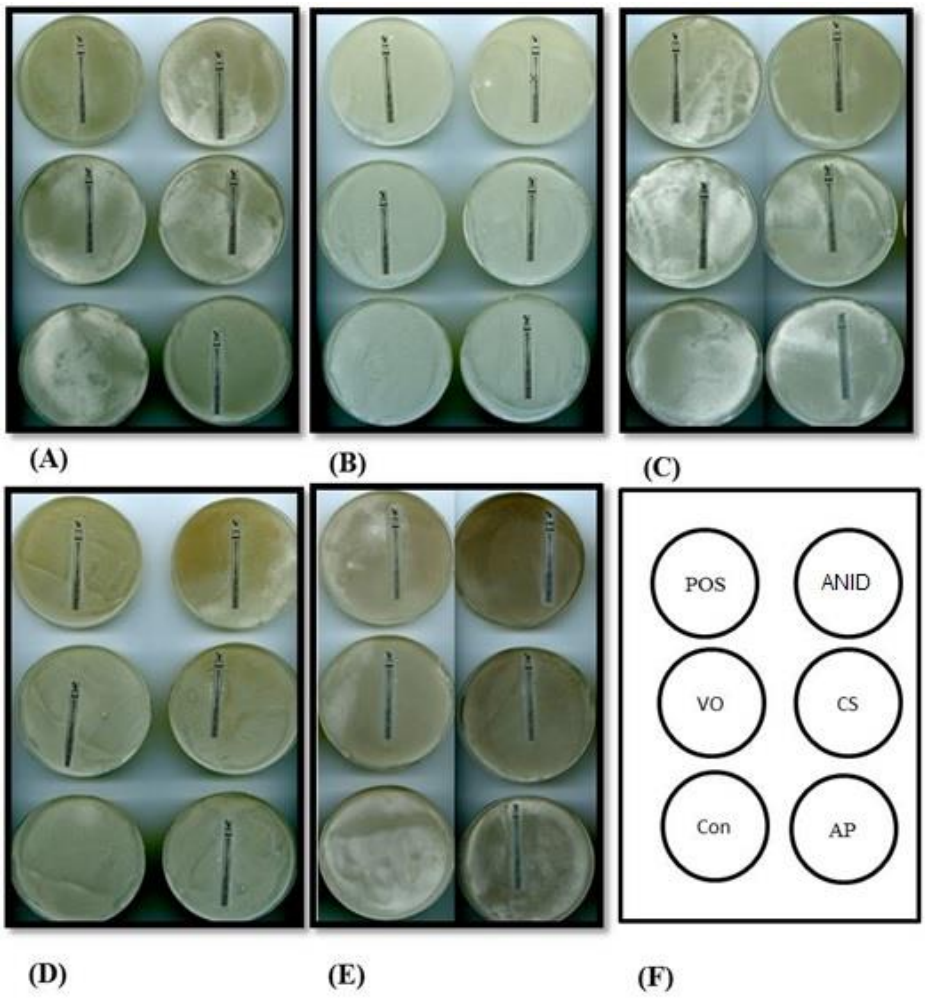

Figure 5.10: Sensitivity of *L. prolificans* to antifungal drugs by using an E-test assay. The E-test strips contained a gradual concentration of antifungal drugs. Strips were placed in the middle of SDA plates, covered with *L. prolificans* spores, and incubated at 30 °C for three days. **A)** Wild-type strain 3.1 **B)** Δ*Lppks1*::*hph* mutant **C)** complemented mutant Δ*Lppks1*::*hph*:*PKS* **D)** mutant Δ*Lpscd1*::*hph* **E)** mutant Δ*Lp4hnr*:: *hph* **F)** An illustrative diagram representing the position of the E-strips on the plates. Fungal growth shows no inhibition zones around the E-test strips, indicating the resistance of *L. prolificans* strains to all antifungal drugs. These results confirm that melanin plays no role in the resistance of *L. prolificans* to antifungal drugs. Images were captured by an Epson Expressi on 1680 Pro scanner. Amphotericin B (AmB), the azoles voriconazole (VOR) and posaconazole (POS), and the echinocandins caspofungin (CASP) and anidulafungin (ANID)

5.3.5.2 Sensitivity of *L. prolificans* strains to amphotericin B by measuring dry weight

The correlation between pigmentation and protection against amphotericin B has already been reported (van Duin, *et al*., 2002). The protective role that melanin plays against amphotericin B was investigated in *Cryptococcus neoformans* by measuring fungal biomass. A concentration of 32 µg/ml of amphotericin B was selected in relation to the proven resistance of *L. prolificans* to a concentration of 16 µg/ml (Kesson, *et al*., 2009). 10^6 spores/ml of *L*. *prolificans* strains were incubated in the 10% TCM containing 32 µg/mL of amphotericin B as a final concentration in TCM flask. A strain of *Aspergillus fumigatus* sensitive to AMB was used as a control. After incubation for three days at 37 °C with shaking, mycelium were collected and dried at 72 °C for two days. Their dry weight was then recorded in mg. The growth of Δ*Lppks1*::*hph* and Δ*Lpscd1*::*hph* mutants increased significantly in comparison to the wild-type strain 3.1, while the Δ*Lppks1*::*hph*:*PKS* complemented strain restored insensitivity to AMB. Similarly, the Δ*Lp4hnr*:: *hph* mutant showed a similar level of insensitivity as the wild-type. Amphotericin B improved their activity by inhibiting *A. fumigatus* growth completely (figure 5.11).

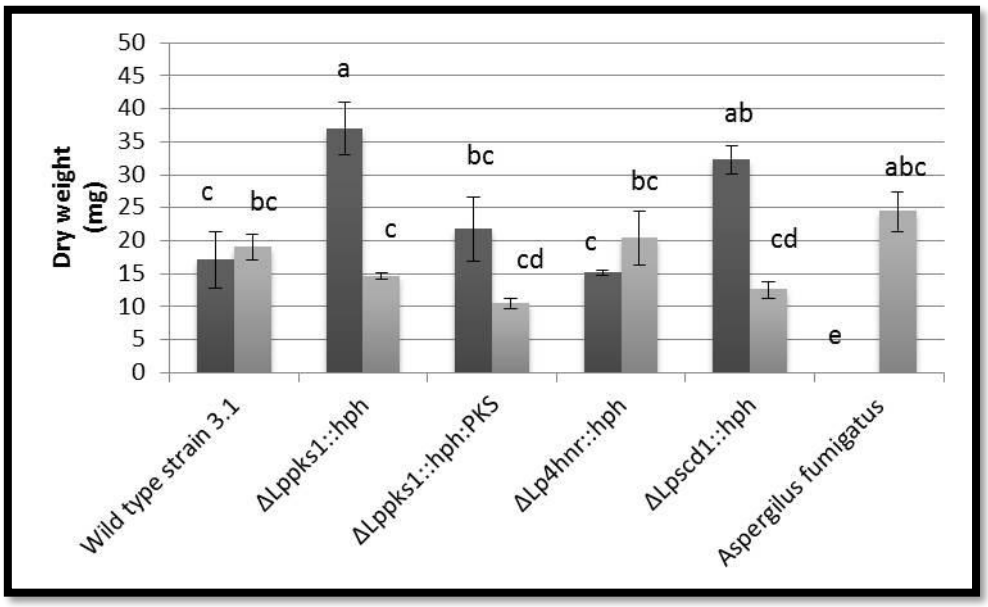

Figure 5.11: Sensitivity of *L. prolificans* strains to amphotericin B. 10^6 spores/ml were inoculated in the TCM flask containing amphotericin B in 10% TCM media and incubated for three days at 37°C. Flasks with MQ and no amphotericin B were used as a control. The light grey bars represent the control samples while the dark grey bars show the average of the three flasks containing amphotericin B. A significant increase in growth was observed in the Δ*Lppks1*::*hph* and Δ*Lpscd1*::*hph* mutants compared to the wild-type strain 3.1. The complemented strain restored insensitivity to a similar level as the wild type. The Δ*Lp4hnr*:: *hph* mutant displayed no sensitivity to amphotericin B at the same level as the wild-type strain 3.1. The different letters show a significant difference ($P< 0.05$). Each bar represents the mean of three replicate samples ± SE.

5.4 Discussion

The aim of this chapter was to investigate the protective role that melanin plays in enabling *L. prolificans* to survive extreme conditions. To achieve this aim, *L. prolificans* strains, melanised and non-melanised, were exposed to different environmental conditions and their survival percentages were calculated relative to control samples (untreated spores). The protective role of melanin against environmental stress was previously confirmed in another species (Nosanchuk, *et al*., 2006), but as of yet there has been no study investigating the protective role of melanin against harsh environmental conditions in the emerging human pathogenic fungus *L. prolificans*. The environmental conditions selected for this study were UV radiation, extreme temperature, oxidative killing by hydrogen peroxide (H_2O_2), and sensitivity to antifungal drugs. Spores aged two weeks are considered mature and could infect humans and animals, therefore this was the age selected in the current study as the experimental time.

The role melanin plays in UVR tolerance has been noticed in human and plant pathogenic fungi (Calvo, *et al*., 2002 ; Allam, *et al*., 2014). It was previously observed that melanin absorbs UVR and prevents damage (Hill, 1992 ; Nosanchuk , *et al*., 2003). The results of this study showed that the survival percentage of the Δ*Lppks1*::*hph* mutant reduced significantly in contrast with its parent strain, whereas a complemented strain of the Δ*Lppks1*::*hph* mutant, Δ*Lp4hnr*::*hph* and Δ*Lpscd1*::*hph* mutants showed no significant difference. It targeted disruption of the *BRM2* gene (gene encodes 1,3,8- trihydroxynaphthalene reductase) in *Alternaria alternata* generated a mutant more sensitive to UVR than the wild-type strain (Kawamura, et al., 1999). Moreover, UV exposure of *alm1* and *brm2-1* mutants (*ALM1* and *BRM2-1* genes encode a polyketide synthetase and a 1,3,8-trihydroxynaphthalene (THN) reductase, respectively) in *Alternaria alternata* resulted in a significant reduction in conidial germination and colony diameter growth in comparison to the melanised wild-type (Kheder, *et al*., 2012). In addition, the melanised human pathogen *Cryptococcus neoformans* improved their resistance to UVR more than the non-melanised strain (Wang & Casadevall, 1994). However, knockdown of the *PKS* gene in *Penicillium marneffei* produced no significant difference in survival percentage with the wild-type after exposure to UVR (Woo, *et al*., 2010). Moriwaki and co-workers found the *PKS1*

gene expression in *Bipolaris oryzae* upregulated in the presence of sunlight by using northern blot analysis (Moriwaki, *et al*., 2004).

Extreme temperature was selected as a second environmental factor in this study. Melanised species *Cryptococcus neoformans* and *Wangiella dermatitidis* show more tolerance than non-melanised strains to extreme temperatures (Rosas & Casadevall, 1997; Jr, *et al*., 2006). In contrast, our results showed no difference in survival percentage between melanised and non-melanised strains following heating or freezing. These results indicate that melanin plays no protective role in *L. prolificans* against extreme temperatures. Numerous physiological mechanisms that protect fungi in low temperatures have been proposed such as lipid content and antifreeze protein (Robinson, 2001). On the other hand, high temperatures have been shown to induce gene expression as a response to high temperature stress (Leach, *et al*., 2013). It could be proposed that there are other factors at play in helping *L. prolificans* to survive extreme temperatures, but not pigmentation.

Melanin is known to be a scavenger of free radicals (Różanowska, *et al*., 1999). This binding property increases tolerance to hydrogen peroxide (H_2O_2) in human fungal pathogens such as *Penicillium marneffei* and *Fonsecaea pedrosoi* (Cunha, *et al*., 2010; Woo, *et al*., 2010). Our results were consistent with previous studies. The survival percentage of Δ*Lppks1*::*hph* and Δ*Lp4hnr*::*hph* mutants decreased significantly after one hour's exposure to H_2O_2 in comparison with the wild-type strain 3.1, while the *ΔLppks1::hph:PKS* complemented strain restored melanin protection to similar levels as the wild type. Moreover, the Δ*Lpscd1*::*hph* mutant displayed no significant difference in survival percentage compared to the wild-type strain. According to this result, melanin's protective role against H_2O_2 damage in *L. prolifcans* depends on the level of pigmentation (melanisation).

It has been shown that *L*. *prolificans* inherently resists antifungal agents amphotericin B, azole, flucytosine and echinocandin (Cuenca-Estrella, *et al*., 1999; Sabatelli, *et al*., 2006; Cortez, *et al*., 2008). However, there has been no molecular genetic study to investigate the mechanism of antifungal resistance in *L. prolificans*. In the current study, E-tests and measurements of fungal dry weight were used to assess the protective role of melanin against antifungal agents.

Several methods have been used to assess microbial susceptibility to antimicrobial drugs (Balouiri, *et al*., 2016). An E-test assay was selected in the current study because this assay is simple to conduct, has no specific requirements, saves time and is consistent with other reliable methods (Brown , *et al*., 1991; Gupta, *et al*., 2015). Our E-test assay results showed that all *L. prolificans* strains, both melanised and non-melanised, are resistant to antifungal agents.

The protective role of melanin against amphotericin B was previously reported in *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Wangiella dermatitidis* (van Duin, *et al*., 2002 ; Jr, *et al*., 2006). It was shown that melanised fungi are more resistant to amphotericin B than non-melanised fungi, because melanin binds amphotericin B and alters the C:N ratio (van Duin, *et al*., 2002 ; Ikeda, *et al*., 2003). Our results showed significant increase in growth in the Δ*Lppks1*::*hph* and Δ*Lpscd1*::*hph* mutants in comparison to the wild type, while the complemented strain of the Δ*Lppks1*::*hph* mutant restored their insensitivity to antifungal drugs to the level of the wild-type strain 3.1. Similarly, the Δ*Lp4hnr*:: *hph* mutant showed no significant difference in growth to the wild-type strain 3.1. A paradoxical growth phenomenon was previously reported in *Candida albicans* and *Aspergillus fumigatus* (Stevens, *et al*., 2004 ; Wiederhold, 2009). This phenomenon was explained as an increase in fungal growth in response to a high concentration of the caspofungin drug, but not to a low or medium concentration (Rueda, *et al*., 2014). The results of this study demonstrate for the first time high growth in response to amphotericin B in the non-melanised strains. This may be a compensatory response related to the loss of pigmentation. The relationship between melanin and chitin has been previously documented (Nosanchuk, *et al*., 2015). It may be that melanin-deficient mutants compensate for melanin loss by increasing chitin content. These results reveal that melanin has no role in protection against antifungal drugs. A previous study that used a melanin-deficient mutant generated by UVR showed consistency with our results (Ruiz-Díez, *et al*., 2003). Also, Polak *et al*. (1989) suggested that melanin in *Wangiella dermatitidis* plays no role in protecting the melanised fungi against antifungal drugs (Polak, *et al*., 1989).

Pooling all these results, it can be concluded that, although melanin protects *L. prolificans* from UVR and oxidative killing by H_2O_2, melanin plays no role in the resistance of *L. prolifican* to conventional antifungal drugs and extreme temperatures.

Chapter 6

The contribution of DHN-melanin to phagocyte survival and pathogenicity of *L. prolificans*

6.1 Introduction

6.1.1 Immunity to fungal pathogens

The majority of the fungi that cause life-threatening disseminated infections of humans are opportunistic environmental moulds that take advantage of an impaired immune system (Parkin, *et al*., 2001). The human immune system, comprising innate and adaptive immunity, is highly effective at preventing fungal infections (Visvanathan, *et al*., 2009), with the innate immune system providing an essential first line of defence against potentially infectious fungal propagules (spores) that are inhaled into the lungs. Innate immune cells, and adaptive cellular and humoral (complement and antibody) immune responses, work together, and in conjunction with antifungal drugs, to prevent invasive fungal diseases of humans (Wont, *et al*., 1990; Nassar, *et al*., 1995).

Innate immune cells comprise macrophages, neutrophils and dendritic cells derived from the myeloid lineage of the hematopoietic stem cell (HSC) (Ibrahim-Granet, *et al*., 2003 ; Gafa, *et al*., 2006; Tarabishy, *et al*., 2008; Geissmann, *et al*., 2010). Neutrophils are granulated polymorphonuclear leukocytes (PMLs) that are recruited from the bloodstream following tissue invasion. In contrast, macrophages, which are derived from monocytes (agranulated leukocytes), are sentinel cells that guard tissues (Erwig, *et al*., 2016). While they differ in morphology and life-span, both macrophages and neutrophils are both phagocytic cells capable of identifying, engulfing and destroying invading pathogens (Erwig, *et al*., 2016). The third type of innate cell is the dendritic cell which plays a critical role in activating adaptive immune defences and further stimulating antigen presenting cells such as macrophages (Banchereau, *et al*., 1998; Erwig, *et al*., 2016). Dendritic cells communicate with $CD4^+$ helper T cells (adaptive immune cells derived from the lymphoid lineage of the HSC), by displaying foreign antigen on Major Histocompatibilty Complex Class II (MHC Class II) proteins on their cell surfaces (Mildner, *et al*., 2014). These displayed antigens are then bound by cognate T cell receptors (TCRs) on $CD4^+$ T cells (Banchereau, *et al*., 1998). Activated

T cells then function to stimulate antibody production by B cells through B cell MHC Class II-TCR interactions and the productions of chemical signalling molecules known as cytokines (Banchereau, *et al*., 1998; Shortman, 2000).

When a fungal pathogen encounters the physical or chemical barriers of the skin or mucosa, it stimulates the complement cascade, and killing by phagocytes (Brakhage, *et al*., 2010; Fábián, *et al*., 2012 ; Owais, *et al*., 2010). Phagocytic cells (macrophages, neutrophils, and dendritic cells) identify fungal spores via cell surface receptors (Herre, *et al*., 2004; Kennedy, *et al*., 2007;Owais, *et al*., 2010), which bind to signature molecules (collectively known as Pathogen-Associated Molecular Patterns (PAMPs) (Peiser, *et al*., 2000 ; Visvanathan, *et al*., 2009) such as mannan and β-glucan present in the fungal cell wall. When these motifs are bound by immune cell Pattern Recognition Receptors (PRRs) such as Dectin-1, β-glucan receptor, mannose receptor, and Toll-like receptors (TLRs), the behaviour of a phagocytic cell, for example an alveolar macrophage, is altered to engulf or phagocytose the spore (Figure 6.1) (Ambrose, 2006). This receptor-mediated interaction between the fungus and the phagocytic cells subsequently induces a signalling cascade that activates adaptive immunity and antibody opsonisation of the spore surface (Mogensen, 2009).

Phagocytic cells express additional cell surface receptors such as complement receptors and Fc receptors that bind to complement proteins (such as C3b) and antibody that opsonise the spore surface, following activation of the lectin complement cascade by mannose-binding lectin (MBL) and antibody-producing B cells respectively (Helmy, *et al*., 2006; Joshi, *et al*., 2006). In addition to MBL, surfactant protein A (SP-A) is also thought to act to opsonise the spore surface (Lillegard, *et al*., 2006; Geunes-Boyer, *et al*., 2009).

Following engulfment and internalisation, the fungal spore is transported into a vacuole elicited from the plasmalemma called a phagosome (Mayorga, *et al*., 1991; Richards, *et al*., 2014). The phagosome then fuses with the lysosome to form the phagolysosome. Within the phagolysosome, the acidic environment combined with lytic enzymes and toxic peroxides then destroy the engulfed spores (Richards, *et al*., 2014; Erwig, *et al*., 2016).

The two intracellular killing mechanisms employed by phagocytic cells to destroy invading pathogens are O_2-dependent and O_2-independent (Weiss, *et al*., 1985). The first mechanism is an oxygen-demanding mechanism (a respiratory burst) (Babior, 2000). Following receptor-mediated activation of the phagocytic cell, superoxide (O_2^-) forms due to the reduction of O_2 by a nicotinamide dinucleotide phosphate (NADPH) oxidase, and the O_2^- then dismutates to hydrogen peroxide (H_2O_2) through the enzyme superoxide dismutase (Babior, 2000). Production of nitric oxide (NO) is catalysed by nitric oxide synthase (Babior, 2000). The H_2O_2 interacts with a halide ion (Cl^-) to produce highly reactive agents (Babior, 2000). The O_2-independent mechanism refers to the killing of pathogens inside the phagocytic cells via enzymes or antimicrobial proteins without O_2 involvement (Catterall J, *et al*., 1986).

6.1.2 Melanin and host immunity

Melanin is a multi-functional material distributed among living organisms (Hill, 1992), and has been shown to alter host immune responses to fungal pathogens. In the dimorphic fungus *Paracoccidioides brasiliensis,* non-melanised mutants are phagocytosed more readily than melanised strains, indicating that melanin interferes with fungal recognition and phagocytosis (da Silva, *et al*., 2006). Similarly, non-melanised strains in *Cryptococcus neoformans* are more sensitive to phagocytic killing than melanised strains (Wang, *et al*., 1995). Treatment of *Fonsecaea pedrosoi* with tricyclazole inhibits melanin biosynthesis and produces strains more sensitive to killing by macrophage compared to the wild-type counterpart (Cunha, *et al*., 2005). Currently, the mechanism by which melanisation confers resistance to macrophage killing, but it is thought that the negative charge of melanin in some way interferes with the phagocytic process (Nosanchuk , *et al*., 2003) as well as it bestowing mechanical strength to the fungal cell wall (Jacobson, 2000).

Macrophages are activated by the fungal cell wall carbohydrate (1-3)-β-D-glucan, inducing inflammatory responses (Kataoka, *et al*., 2002). Melanin plays an important role by masking this PAMP from recognition by pattern recognition receptors PRRs on the macrophage cell surface (Chai , *et al*., 2010). In *Aspergillus fumigatus,* melanin is

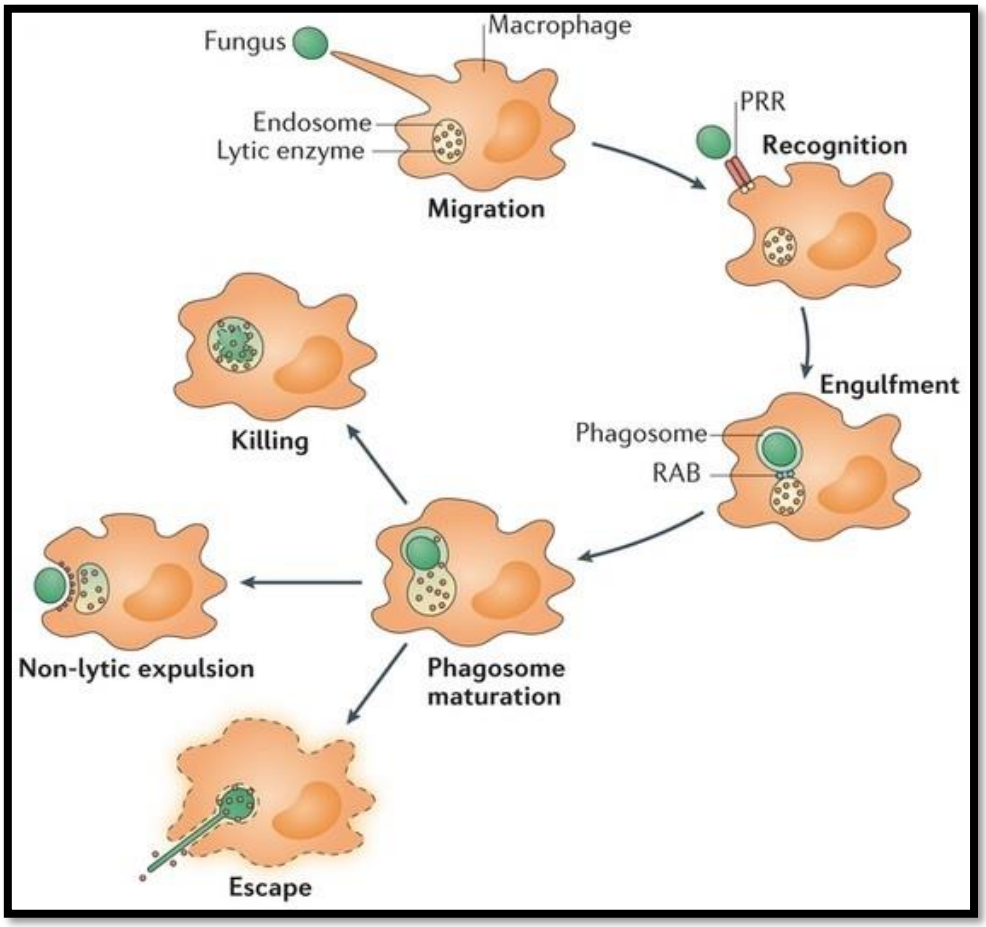

Figure 6.1: Stages in macrophage phagocytosis of a fungal spore. The interaction of a macrophage and a fungal spores initiate with chemotactic attraction and recognition of fungal PAMPs by PRRs on the surface of the macrophage. This triggers internalisation of the spore and delivery to the phagosome. The phagosome in turn fuses with the lysosome (to form the phagolysosome) mediated by Rab GTPases, where the engulfed spore is eventually destroyed or degraded, and then released extracellularly via exocytosis or released intracellularly to undergo further processing. Some fungi are able to evade macrophage killing and the spores germinate and escape from the phagocyte. Figure taken from (Erwig, *et al*., 2016).

an immunologically inert shroud on the conidial cell surface, preventing dendritic cell activation (Bayry, *et al*., 2014)**,** and reducing acidification of phagolysosome (Thywißen, *et al*., 2011). Polyketide synthase deficient mutants that have abnormal melanisation are more susceptible to phagocytosis (Thywißen, *et al*., 2011), while melanin decreases acidification by inhibiting vATPases (Thywißen, *et al*., 2011).

Melanin quenches reactive oxygen species (ROS) (Heinekamp, *et al*., 2012), and protects fungal pathogen by scavenging ROS (Cunha, *et al*., 2010). The protective role of melanin against hypochlorite, permanganate and hydrogen peroxide has been investigated in *Cryptococcus neoformans* (Jacobson, *et al*., 1993), with melanin bestowing resistance to hypochlorite and permanganate but not hydrogen peroxide. Moreover, the survival of *Cryptococcus neoformans* melanised strain is greater than non-melanised mutants in the epinephrine oxidative system (Polacheck, *et al*., 1990).

The current hypothesis advocates melanin as a physical shield against the host immune system, prolonging survival of fungal pathogens and enabling dissemination throughout the body (Jacobson, *et al*., 1993; Volling, *et al*., 2011; Eisenman, *et al*., 2012; Bayry, *et al*., 2014). In addition, melanin may act as an immune modulator (Nosanchuk, *et al*., 1998; Alviano, *et al*., 2004; Mednick, *et al*., 2005) and activator of the alternative pathway of the complement system (Rosas, *et al*., 2002).

6.1.3 Aims of Chapter 6

In this chapter, I set out to investigate the role that melanin plays in protecting *L. prolificans* from killing by macrophages, innate immune cells that provide the primary front-line defence against opportunistic fungal pathogens of the lung (Babior, 2000). To enable visualisation of the phagocytic process, I describe the generation of mutants of the wild-type *L. prolificans* strain 3.1 and melanin-deficient albino mutant Δ*Lppks1*::*hph* (Chapter 3) expressing green fluorescent protein (GFP). I investigate the role of melanin in macrophage phagocytosis by determining the phagocytic index (Pi) and percentage phagocytosis (P%) of the melanised and albino GFP mutants, and establish the role of melanin in pathogen survival by determining spore germination following phagocytosis. Finally, I use the model invertebrate *Galleria mellonella* (Wax moth) to the study the role of melanin in host infection by the pathogen.

6.2 Material and methods

6.2.1 Maintenance of macrophage J774A.1 cell line

The adherent macrophage cell line J744A.1 was obtained from ATCC (ATCC®TIB-67™). The cell line was original generated from a female BALB/cN mouse with reticulum cell sarcoma (https://www.lgcstandards-atcc.org/Products/All/TIB-67.aspx?geo_country=gb). The cell line was stored long-term in liquid nitrogen. For culture, cells were defrosted and cultured in 75 cm^2 tissue culture flasks containing 10 mL of pre-warmed 10% tissue culture medium (Section 2.2.1). Flasks were incubated in a Sanyo CO_2 incubator (MCO-17A) at 37^oC and 5% CO_2. Sub-culturing was carried out every 2 days. Adherent cells were dislodged by using a cell scraper (C5981-100EA: Sigma-Aldrich), aspirated into 15 mL Falcon tubes (62.554.502: Sarstedt), and washed three times by suspension and centrifugation at 14 000 x g for 5 min each time and re-suspension in fresh TCM. Two-mL of washed cell suspension containing 10^5 cells/mL (determined using a haemocytometer) were then used to seed flasks containing fresh TCM for culture under the conditions described.

6.2.2 Generation of ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP transformants

The vector pCB1532 (Sweigard, et al., 1997), which contains the sulfonylurea resistant allele and ToxA-GFP fragment (Figure 6.2), were transformed into the *L. prolificans* wild-type strain 3.1 and Δ*Lppks1*::*hph* mutant using the fungal protoplast transformation described in Chapter 2, Section 2.3. Putative ToxA-GFP (wild-type) and Δ*Lppks1*::*hph*:ToxA-GFP (albino, PKS-deficient mutant) transformants expressing GFP were selected based on resistance to sulfonyl urea using BDCM medium supplemented with 100 µg/mL of chlorimuron ethyl.

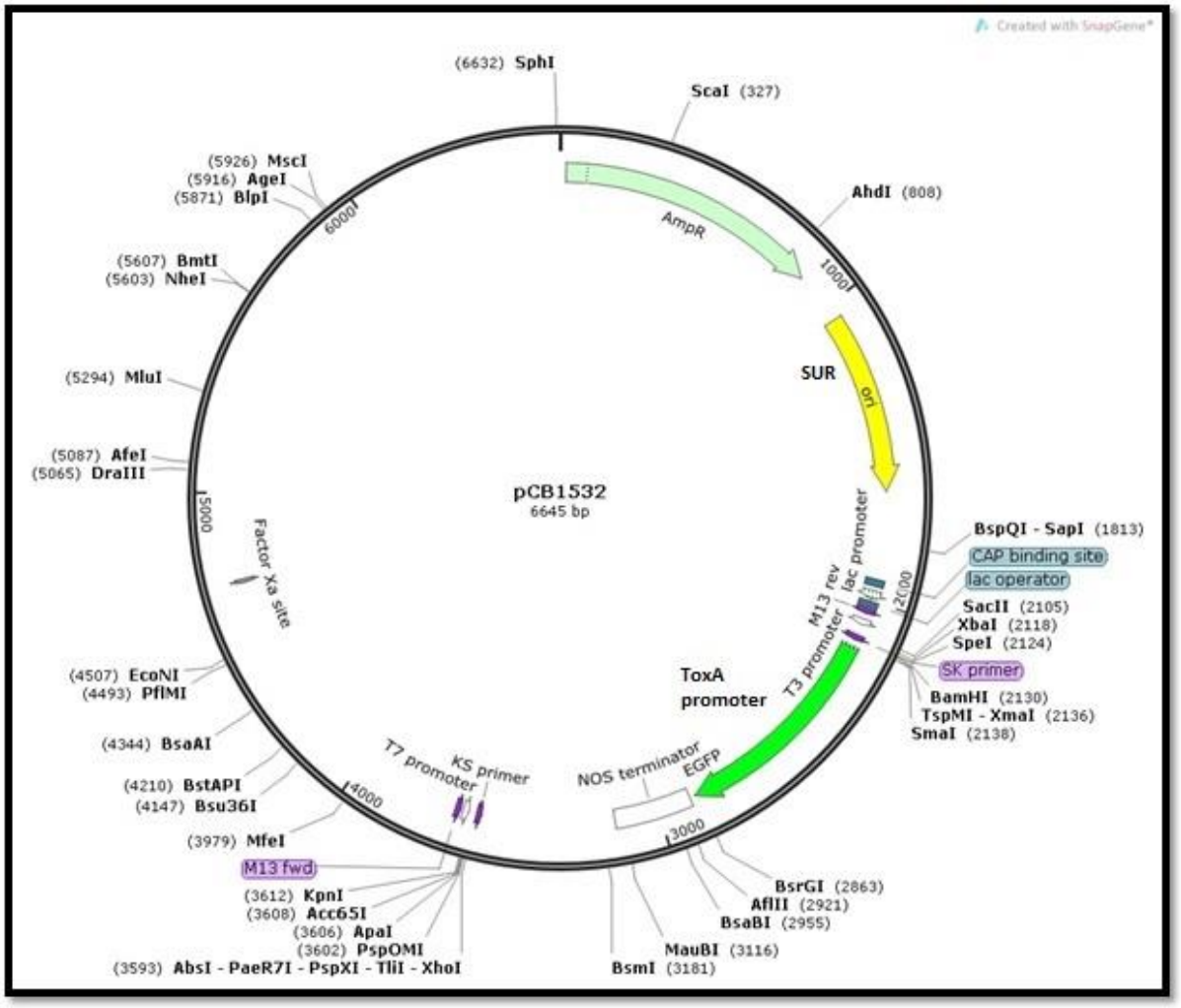

Figure 6.2: Schematic representation of the pCB1532 vector. The vector contains the sulfonylurea gene and the EGFP gene under the control of the *Pyrenophora tritici-repentis* ToxA gene promoter. The EGFP gene encodes a GFP variant that contains a serine-to-therionine substitution amino acid 65, in addition to 190 silent base mutations corresponding to a codon in human protein, which is used preferentially. ToxA-GFP was cloned in the position of a multiple cloning site (ClaI/EcoRI).

6.2.3 Confirmation of GFP expression in wild-type ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP transformants

6.2.3.1 GFP expression

Spores of the mutants were harvested and washed three times as described before (Chapter 2, Section 2.2.1). Ten-µl of washed conidial suspension were placed onto a glass slide, overlaid with a coverslip, and examined using an IX81 motorized inverted microscope (Olympus, Hamburg, Germany) to visualize green fluorescence of spores.

6.2.3.2 PCR and sequencing of ToxA-GFP fragment

Genomic DNA was isolated according to the DNA extraction procedure described in Chapter 2, Section 2.2.1. ToxA-GFP fragments were amplified by PCR using the primer pairs GFPs-F1 (5'-TCCCGTCCTATCTAACAATC-3')/GFPs-R1 (5'-ATGTGATCGCGCTTCTCGTT-3') or ToxA-GFP-F (5'-TAGAACTAGTGGATCACGTCGACGGTATCGATTGG-3')/ ToxA-GFP-R (5'-CGGTATCGATAAGCTCTCATGTTTGACAGCTTATC-3'). PCR cycling conditions involved: initial denaturation 98 °C for 3 min followed by PCR cycling parameters of 98 °C for 1 min, 55 °C for 1 min and at 72 °C for 1 min/Kb target length for 35 cycles, followed by a final extension at 72°C for 10 min. The primer pair ToxA-GFP-F/ToxA-GFP-R was designed to include 15-bp of sequence homologous to the ends of the linearized vector pCB1532. This extension was added to enable ligation of the ToxA-GFP fragment to the vector pCB1532. However, the pCB1532, which containing ToxA-GFP fragment and sulfonyl urea resistant allele, already found in the lab and used to integrate the ToxA-GFP fragment into the wild-type strain 3.1 and PKS-deficient mutant. PCR products were amplified with the primer pair GFPs-F/GFPs-R and purified by using Wizard® SV gel and PCR Clean-Up Systems (Promega) (see Chapter 2, Section 2.2.2.3). The purified products were send to MWG Eurofins https://eurofinsgenomics.eu/ for sequencing. Sequencing was carried out by using cycle sequencing technology (dideoxy chain termination / cycle sequencing) on ABI 3730XL sequencing machines.

6.2.4 Quantification of phagocytosis percentage (%), phagocytic index (Pi), and germination inhibition of ToxA-GFP and Δ*Lppks1*::*hph:*ToxA-GFP transformants

The wells of µ-Slide 8 well slides (ibiTreat, Germany) were inoculated with 10^5 J774A.1 macrophage/well and incubated for 24 h at 37 ^oC and 5% CO_2. Conidia of *L. prolificans* were harvested, washed three times with sterile filtered 1% PBS, and finally re-suspended in warmed 10% TCM. Slide containing adherent macrophages were washed three times with pre-warmed 10% TCM and 10^6 conidia introduced into each well to give a macrophage:spore ratio of 1:10. The slides were incubated at 37 ^oC and 5% CO_2 for 4 h to allow the conidia to be engulfed by the macrophage cells, after which the bathing solution containing free spores was removed, and the cells washed three times with warmed 10% TCM. Phagocytosis was then quantified by examining five randomly selected sites with an IX81 motorized inverted microscope (Olympus, Hamburg, Germany) fitted with 488 nm excitation and 509 nm emission filters, with a total of 150 macrophage cells counted per well.

Phagocytic percentage (P%) and phagocytic index (Pi) were then calculated for each treatment using the formula shown below (Gil-Lamaignere , et al., 2001), where P% is the percentage of cells containing conidia and Pi is the average number of phagocytosed conidia per phagocytosing cell. *A, B, C D E, F* represent the number of macrophage cells with no conidia, with one conidium, two conidia, three conidia, four conidia, and with more than four conidia, respectively. Conidia that remained attached to macrophages after three washes with 1XPBS, but had been 50% ingested, were considered to have been phagocytosed. The experiment was repeated three times with three replicates.

$$\textbf{Percentage of phagocytosis} = 100\left(\frac{B+C+D+E+F}{A+B+C+D+E+F}\right)$$

$$\textbf{Phagocytic index} = \left(\frac{B+2c+3D+4E+5F}{B+C+D+E+F}\right)$$

For germination inhibition experiments, slides were prepared as described and incubated for 6 h at 37 ^{o}C and 5% CO_2. Slide wells were washed three times with 1X PBS and examined using the IX81 motorized inverted microscope (Olympus, Hamburg, Germany) and the number of germinated conidia enumerated by examining 150 macrophage cells selected at random as described. The experiment was repeated three times each with three replicates. The percentage inhibition of spore germination was determined by using the formula (Gil-Lamaignere, *et al*., 2001):

Percentage inhibition of spore germination = (non-germinated conidia/total conidia counted) x 100

6.2.5 Determination of killing of *L. prolificans* spores by J774A.1 macrophages

6.2.5.1 Mycological culture

Macrophage cells were dislodged by using a cell scraper, and washed three times with pre-warmed 10% TCM by repeated suspension and centrifugation at 14 000 x g for 5 min. Cell concentrations were determined using a haemocytometer and $2x10^5$ cells/well were seeded into 96-well cell culture plates (655180: Greiner bio-one) and incubated in a Sanyo CO_2 incubator (MCO-17A) at 37 ^{o}C and 5% CO_2 for 24 h. Wells were washed three times using warmed 10% TCM, and 10^6 washed spores (Chapter 2, Section 2.2.1) suspended in warmed 10% TCM were applied to the macrophage cells for 6, 8, 10 h to allow phagocytosis. The wells were then washed three times with 1X PBS to remove non-adherent, non-phagocytosed conidia. The adherent macrophages were lysed with 200 µl of lysis buffer (0.025% SDS dissolved in 1X PBS) for 3 min, and released spores washed three times with 1X PBS to remove SDS. The spore concentration was adjusted to 10^3 spores/ml were and 300 µl aliquots spread on replicate SDA plates. The plates were incubated for 3 d at 30 ^{o}C to allow spore germination and formation of countable colonies.

6.2.5.2 MTT assay

Killing of spores by macrophages was also determined by using the dye MTT (M 2128-G: Sigma-Aldrich), which enables the metabolic activities of cells to be determined colorimetrically. Macrophages cells ($2*10^5$) were seeded into the wells of 96-well culture plates (Greiner bio-one) and incubated for 24 h in a Sanyo CO_2 incubator (MCO-17A) at 37 ^oC and 5% CO_2. One hundred-µl of washed spore suspension containing 10^6 spores were added to the macrophages for 6, 8, and 11 h, after which the macrophages were washed three times with 1X PBS to remove non-phagocytosed spores. Macrophage cells were lysed with 200 µl of lysis buffer (0.025% SDS dissolved in 1X PBS) for 3 min and the spores were aspirated, washed three times with 1X PBS, and re-suspended in 200 µl of RPMI-1640 containing 0.125 mg/ml of the dye MTT in 96-well microtiter plates. The spore suspensions were incubated in a Sanyo CO_2 incubator (MCO-17A) at 37 ^oC and 5% CO_2 for 20 h, after which the plates were centrifuged to precipitate insoluble components. The supernatants were removed, and precipitates solubilised with 200 µl of extraction solution (isopropanol containing 5% HCl) for 30 min at room temperature. Optical density (OD) was measured in a spectrophotometer (TECAN, infinite M200 PRO, Nano Quant) at 570 nm.

Percentage killing of spores was determined using following the equation (Levitz, *et al*., 1985):

Percentage of killing = 1 - (A_{570nm} of spores incubated with phagocyte cells/A_{570nm} of spores incubated without phagocyte cells) x 100.

6.2.6 Pathogenicities of melanin mutants in *Galleria mellonella* (Wax moth)

Galleria mellonella larvae were purchased from a commercial supplier (http://www.livefoodsdirect.co.uk/Category/Waxworms), and were maintained on wood chips at 15 ^oC in the dark for 3 d until used. Preliminary investigations set out to determine the appropriate concentration of *L. prolificans* spores for larval infection studies. To this end, larvae were injected with various spore concentrations including 10^3, $3x10^3$, $5x10^3$, 10^4, $3x10^4$, $5x10^4$, 10^5, $3x10^5$, $5x10^5$, and 10^6 spores/ml. Spores were harvested and washed three times with 1X PBS, and 10 µL volumes injected into the haemocoel of 10 larvae by using a Hamilton syringe. The same volume of 1X PBS

was injected into larvae as a control, and 10 larvae were incubated without injection. The larvae were incubated in the dark at 37 ^{o}C, and the number of melanised larvae counted over a 10 d period.

6.3 Results

6.3.1 Generation of ToxA-GFP and *ΔLppks1*::*hph*:ToxA-GFP transformants

Protoplasts of the *L. prolificans* wild-type strain 3.1 and albino PKS-deficient mutant Δ*Lppks1*::*hph* were transformed with the vector pCB1532 (Sweigard, *et al*., 1997) (Figure 6.2), re-generated in BDCM media containing 300 µg/ml of sulfonylurea. Putative transformants were then sub-cultured on BDCM supplemented with 100 µg/ml of sulfonylurea; putative transformants of both strains grown under these conditions are shown in Figure 6.3 A and B. BCDM cultures grown under sulfonylurea were thereafter grown on OA (Figure 6.3 C and D respectively) where they assumed the typical growth and pigment characteristics of the 3.1 and Δ*Lppks1*::*hph* counterparts (melanisation in the ToxA-GFP strain and albinism in the *ΔLppks1*::*hph*:ToxA-GFP strain).

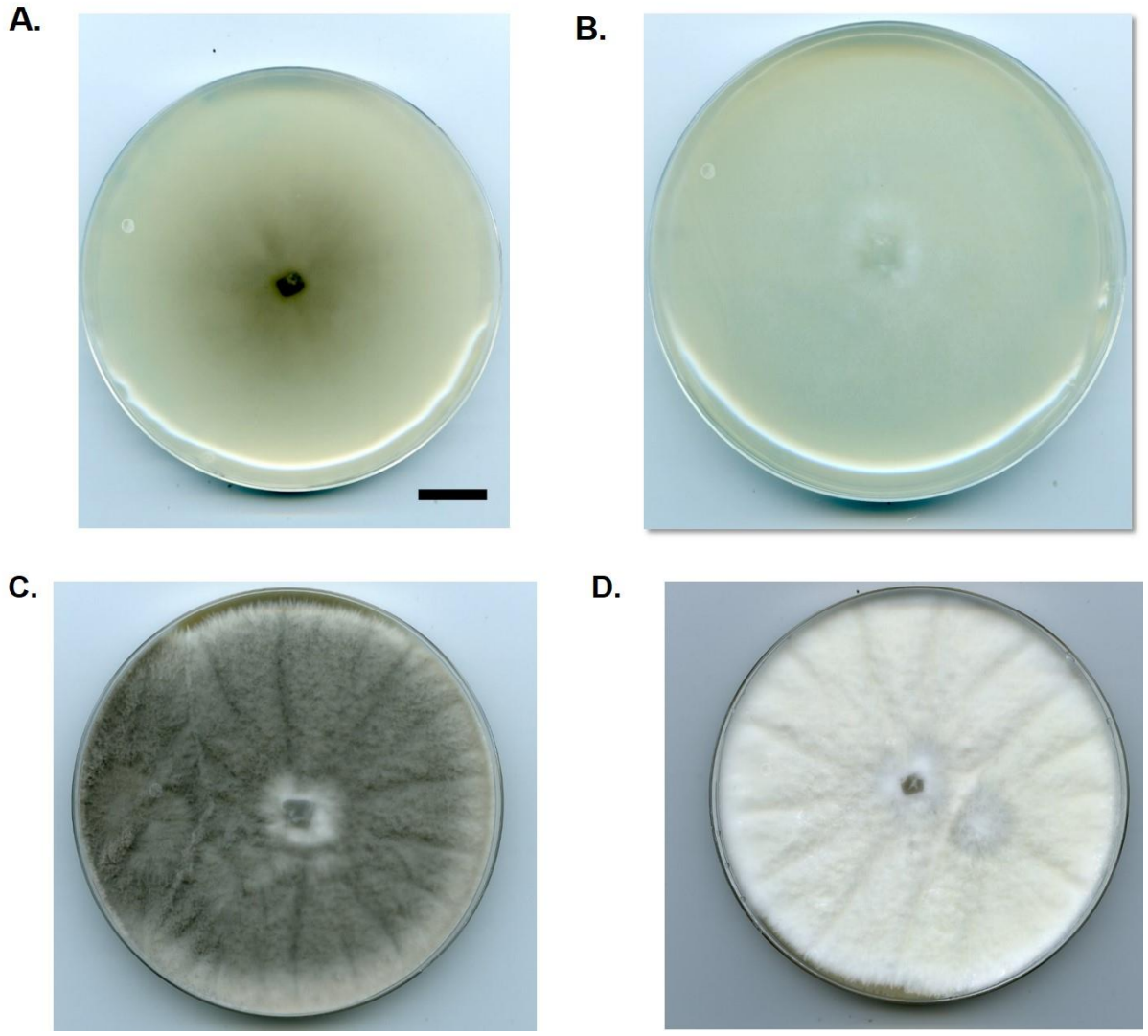

Figure 6.3: Growth of ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP putative transformants on BDCM-sulfonylurea selection medium and OA. Putative transformants were sub-cultured onto BDCM containing 100 µg/ml of sulfonylurea (A and B) and OA (C and D) and incubated at 30 ◦C for 2-weeks in the dark. **(A)** ToxA-GFP; **(B)** Δ*Lppks1*::*hph*:ToxA-GFP; **(C)** ToxA-GFP; **(D)** Δ*Lppks1*::*hph*:ToxA-GFP. Scale bar that applied to all images = 1.5 cm.

6.3.2 PCR and sequencing to confirm integration of the GFP fragment into the ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP mutants.

Integration of the GFP fragment into ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP putative mutants was confirmed by PCR amplification by using two different primer pairs (GFPs-F/GFPs-R and ToxA-GFP-F/ToxA-GFP-R), designed using the ToxA promoter and GFP nucleotide sequences shown in Figure 6.4.

ACGTCGACGGTATCGATTGGAATGCATGGAGGAGTTCTGTACGCGCAATTCCGCTCTCCGTAAGGATGCTTCGGAGGTGCACA
TGGTCTCATACATGTAGGCCCGACGAGGATCGAGTCGGTTCCGAAGTAGGATCGTCTCGATTGTTGGGCATCATTGCATGGAC
ATTCAGAGGGCCTACTGATACCTGGAATCCGCACCGTCCGGCTACCTAGCAATAAGATTCTGTGTATATAAAGGGCTAAGGTG
TCCGTCCTTGATAAAACCACCACCCTCAACAACTTACCTCGACTATCAGCATCCCGTCCTATCTAACAATCGTCCATCGGTAT
CCAACTCCAACTCTATTCGCAGGGTCCTAGAATCGTAAGTACACGCTTATATCTTGTTGCCAGCGATAGCTGACATTGAATGA
ATAT**AGTCCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGG**
CCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGCACCTGAAGTTCATCTGCACCACCGGCA
AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAG
CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAA
GACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCA
ACATCTTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAG
GTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGA
CGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACA
TGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCACGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCCCGGCTGCA
GATCGTTCAAACATTTGGCAATAAAGTTTCTTAAGATTGAATCCTGTTGCCGGTCTTGCGATGATTATCATATAATTTCTGTT
GAATTACGTTAAGCATGTAATAATTAACATGTAATGCATGACGTTATTTATGAGATGGGTTTTTATGATTAGAGTCCCGCAAT
TATACATTTAATACGCGATAGAAAACAAAATATAGCGCGCAAACTAGGATAAATTATCGCGCGCGGTGTCATCTATGTTACTA
GATCCGATGATAAGCTGTCAAACATGAG

Figure 6.4: Nucleotide sequences of EGFP gene under the control of *Pyrenophora tritici-repertis* ToxA gene promoter. The open reading frame of EGFP is shown in bold and highlighted in yellow colour while *Pyrenophora tritici-repertis* ToxA gene promoter is highlighted in grey. Two primers pairs were used to amplify the ToxA-GFP fragment was highlighted in green colour while another primer pair was highlighted in red colour.

Gel electrophoresis confirmed successful integration of ToxA-GFP into the genomes of ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP (Figure 6.5 A and B). The PCR products of (Figure 6.5 A) were purified and sequenced to confirm insertion of the ToxA-GFP construct into the genomes of ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP (Figures 6.6 and 6.7).

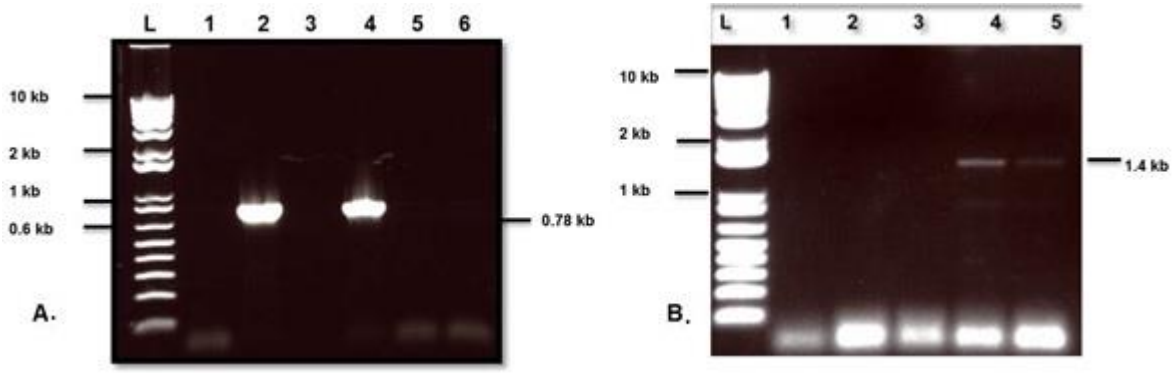

Figure 6.5: Confirmation of ToxA-GFP integration into fungal genomes. PCR amplicons were separated in 0.8% agarose gels and stained with ethidium bromide. **(A)** PCR amplicons amplified with the primer pair GFPs-F/GFPs-R. Lane L is the 1-kb ladder, while lanes 1-6 are control, ToxA-GFP, no DNA, Δ*Lppks1*::*hph*:ToxA-GFP, wild-type 3.1, and the PKS-deficient mutant Δ*Lppks1*::*hph*, respectively. The PCR product of 0.78-kb in lanes 2 and 4 indicate successful integration into the genomes of ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP, and absence in the untransformed 3.1 and Δ*Lppks1*::*hph* negative controls. **(B)** PCR amplicons amplified with the primer pair ToxA-GFP-F/ToxA-GFP-R. Lane L is the ladder, lane 1 is the wild-type strain 3.1, lane 2 is the albino mutant Δ*Lppks1*::*hph*, lane 3 is control, while lanes 4 and 5 are mutants Δ*Lppks1*::*hph*:ToxA-GFP and ToxA-GFP respectively. The PCR product of 1.439-kb (whole fragment) in lanes 4 and 5 indicate successful integration of ToxA-GFP in the genomes of the Δ*Lppks1*::*hph*:ToxA-GFP and ToxA-GFP mutants.

6068199;Value Read;;wr;AAA0058415;premix w.temp;final result;730;">wr_premix -- 11741 of sequence
CGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGG
TGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCT
TGAAGTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGT
TGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCT
TCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGT
AGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGG
ACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCGT
AGGTGAAGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGA
ACTTCAGGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAACT
TGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCT
CCTCGCCCTTGCTCACCATGGCCTATATTCATTCATTGTCAGCTATCGCTGGCAACAAGA
TATAAGCGTGTACTTACGATTCTAGGACCCTGCGAATAGAGTTGGAGTTGGATACCGATG
GACGATGTAGA";
6068199;Value Read;;wf;AAA0057256;premix w.temp;final result;732;">wf_premix -- 15747 of sequence
ACTCTATTCGCAGGGTCCTAGAATCGTAAGTACACGCTTATATCTTGTTGCCAGCGATAG
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AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCTTCACCTACGGCGTGCAGTGCTTCA
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ACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGG
TGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGG
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TCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCG
AGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCC
CCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCA
ACGAGAACGCGGA";

Figure 6.6: Nucleotide sequences of ToxA-GFP integrated into ToxA-GFP transformant. ToxA-GFP fragments were purified and sequenced to confirm successful integration of ToxA-GFP into the genomic DNA of ToxA-GFP transformant. The sequencing was performed to identified ToxA-GFP fragment size 0.78 kb.

6121032;Value Read;;PKGFR;AAA0057263;premix w.temp;final result;730;">PKGFR_premix -- 9739 of sequence
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GACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGCACGCCG
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TTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGC
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GGACGATTGTA";
6121032;Value Read;;PKGFF;AAA0057262;premix w.temp;final result;731;">PKGFF_premix -- 9740 of sequence
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CTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGA
GGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAA
GGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTA
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CGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGG
CCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCC
CAACGAAGGGGG";

Figure 6.7: Nucleotide sequences of ToxA-GFP integrated into Δ*Lppks1*::*hph*:ToxA-GFP transformant. ToxA-GFP fragments were purified and sequenced to confirm successful integration of ToxA-GFP into the genomic DNA of Δ*Lppks1*::*hph*:ToxA-GFP transformant. The sequencing was performed to identified ToxA-GFP fragment size 0.78 kb.

6.3.3 Expression of green fluorescent protein

Examination of spores of the ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP mutants showed green fluorescence, demonstrating constitutive expression of GFP (Figure 6.8). Green fluorescence was not observed in the untransformed wild-type 3.1 and Δ*Lppks1*::*hph* counterparts (not shown).

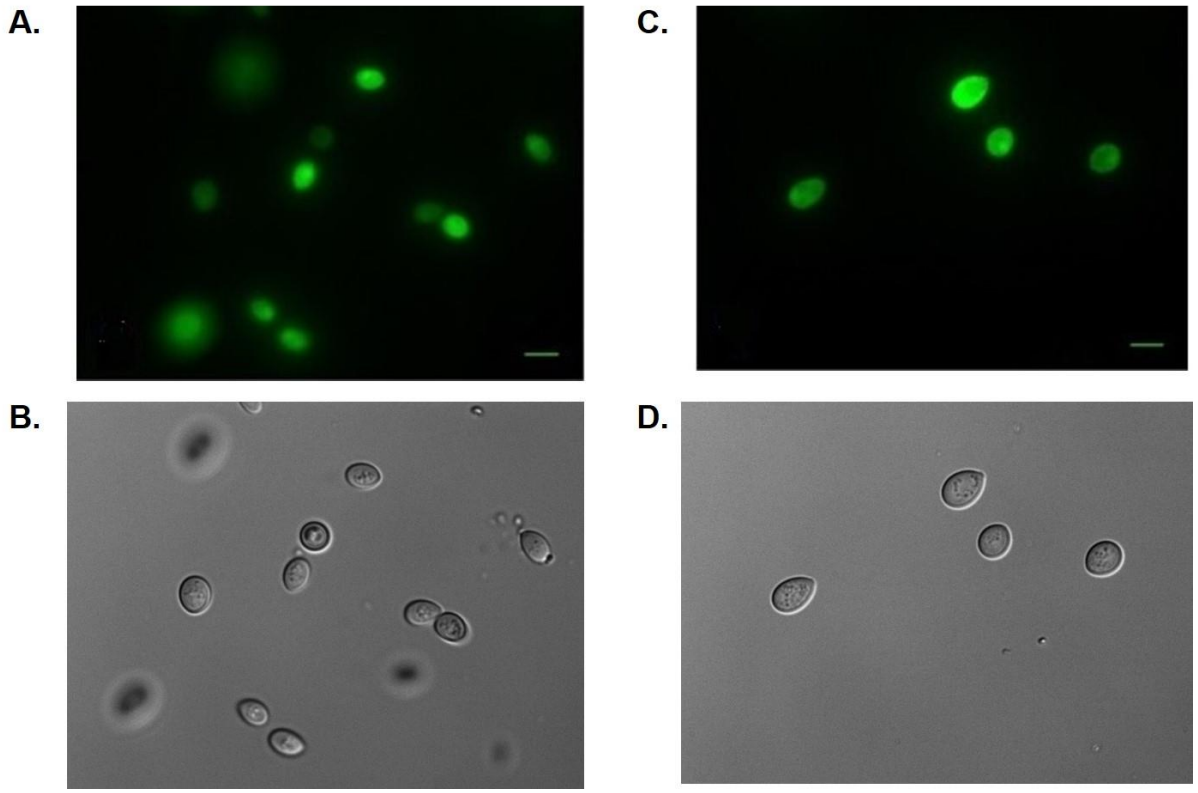

Figure 6.8: Photomicrographs showing GFP expression in spores of the mutants ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP. A) Spores of strain ToxA-GFP showing green fluorescence. **B)** Same image as A but examined under bright field microscopy. **C)** Spores of strain Δ*Lppks1*::*hph*:ToxA-GFP showing green fluorescence. **D)** Same image as C but examined under bright field microscopy. Scale bars in A and C = 10 µm.

6.4 Phenotypic characterisation of ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP mutants

6.4.1 Hyphal growth and sporulation

Hyphal growth of *L. prolificans* 3.1, Δ*Lppks1*::*hph*, ToxA-GFP, and Δ*Lppks1*::*hph*:ToxA-GFP were similar over a 2 week growth period on OA (Figure 6.9). At day 14, there were no significant differences in colony diameters of the four strains. Spore concentrations were quantified at day 14 using a haemocytometer and showed no significant differences between the four strains (Figure 6.10).

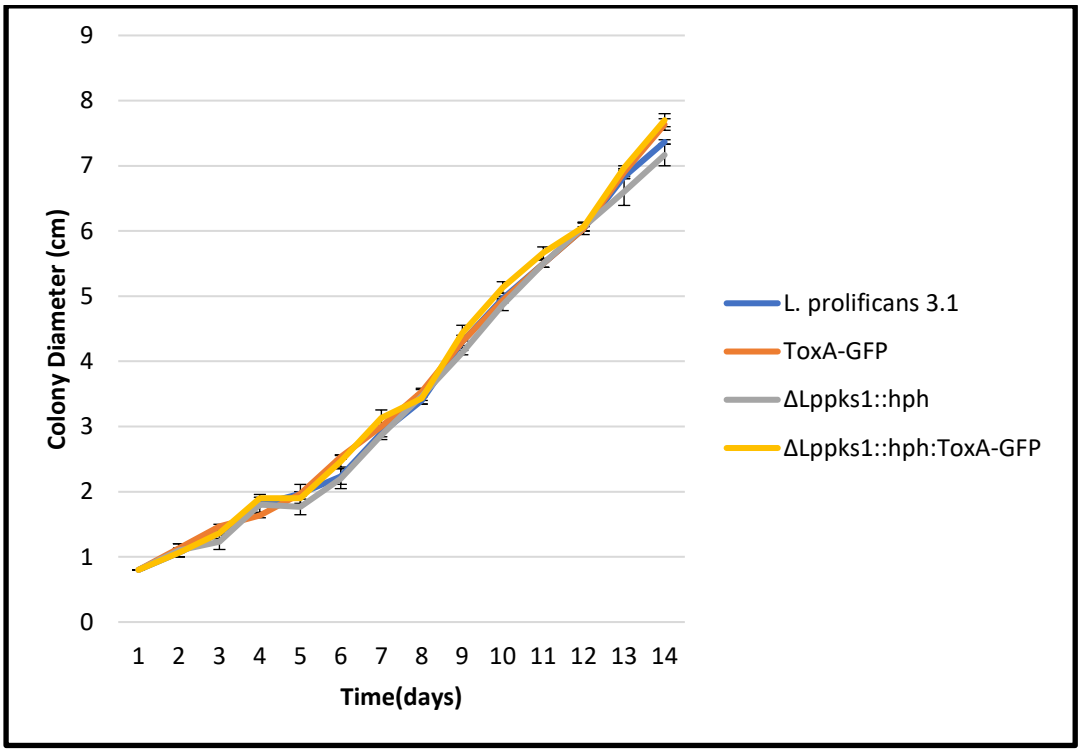

Figure 6.9: Colony diameters of fungi grown on OA over 14 days. *L. prolificans* strains were sub-cultured onto OA plates and incubated at 30 °C for 14 d. Colony diameters were measured daily and showed no significant differences between strains (P>0.05; Student's t-test). Each point is the mean of 3 replicates ± standard error, and experiments were conducted three times.

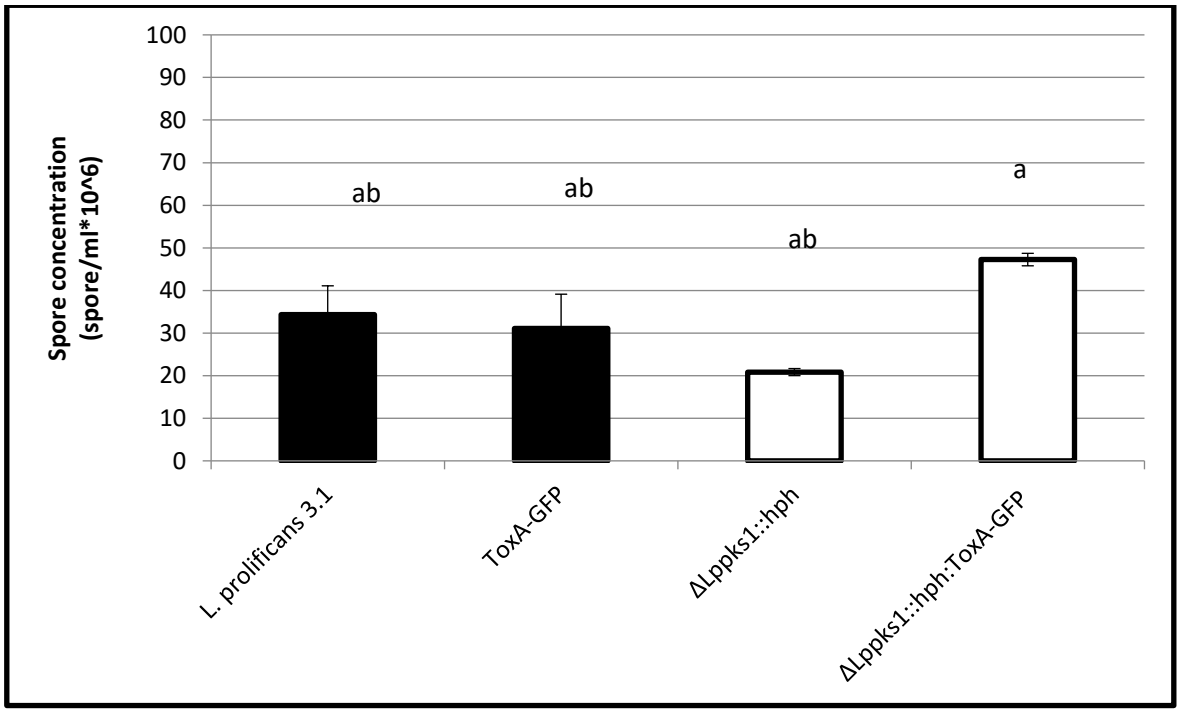

Figure 6.10: Evaluation of spore concentrations (spores/ml) of *L. prolificans* strains on OA. Spores were harvested from 14-day-old OA plate cultures and concentrations determined using a haemocytometer. There was no significant difference in spore production between the four strains. Bars with the same letters are not significantly different to one another at $P>0.05$. Each bar is the mean of three replicates ± standard error, and experiments were conducted three times.

6.5 Percentage phagocytosis (P%)

The role of melanin in preventing phagocytosis of *L. prolificans* spores by J774A.1 macrophages was determined by measuring phagocytosis percentage (%). It was hypothesised that melanin protects spores of the fungus from phagocytosis by preventing recognition by macrophages. To better visualise spore phagocytosis, a GFP-expressing mutant of the melanised wild-type strain 3.1 (ToxA-GFP), and a GFP-expressing strain of the albino PKS-deficient mutant Δ*Lppks1*::*hph*:ToxA-GFP, were used (Figure 6.11). Using these strains, no significant differences in P% (defined as the % of macrophage cells containing engulfed spores) were found, with values of 90.4±1.8 and 86.2±5.6 for melanised ToxA-GFP and non-melanised *ΔLppks1*::*hph*:ToxA-GFP conidia, respectively (Figure 6.12).

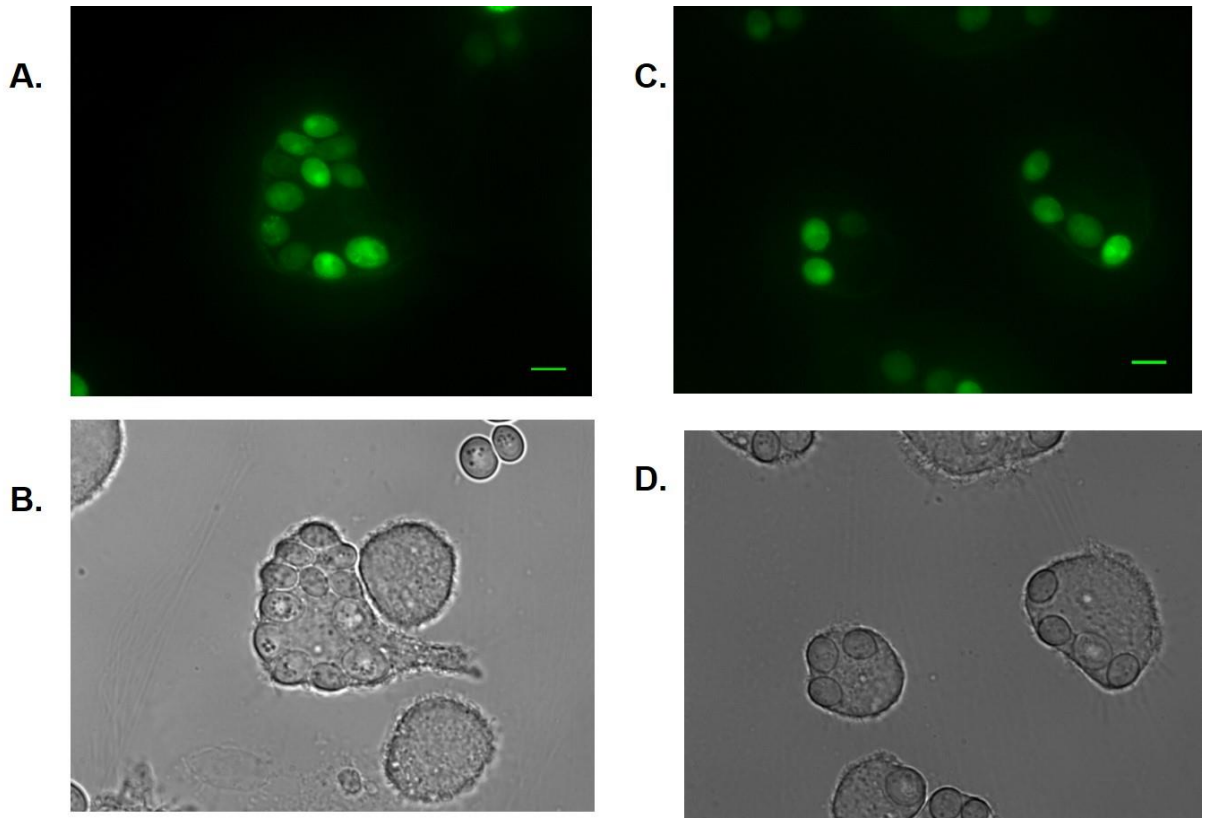

Figure 6.11: Photomicrographs showing conidia of *L. prolificans* strains phagocytosed by the microphage cell line J774A.1. Spores were incubated with macrophage cell at 37 ^{o}C and 5% CO_2 for 4 h. Green fluorescent spores of the wild-type GFP mutant ToxA-GFP **(A)** and Δ*Lppks1*::*hph*:ToxA-GFP **(C)** can be seen within macrophage cells. Bright field images of engulfed spores are shown in **B** and **D**, respectively. Scale bars in A and C = 10 μm.

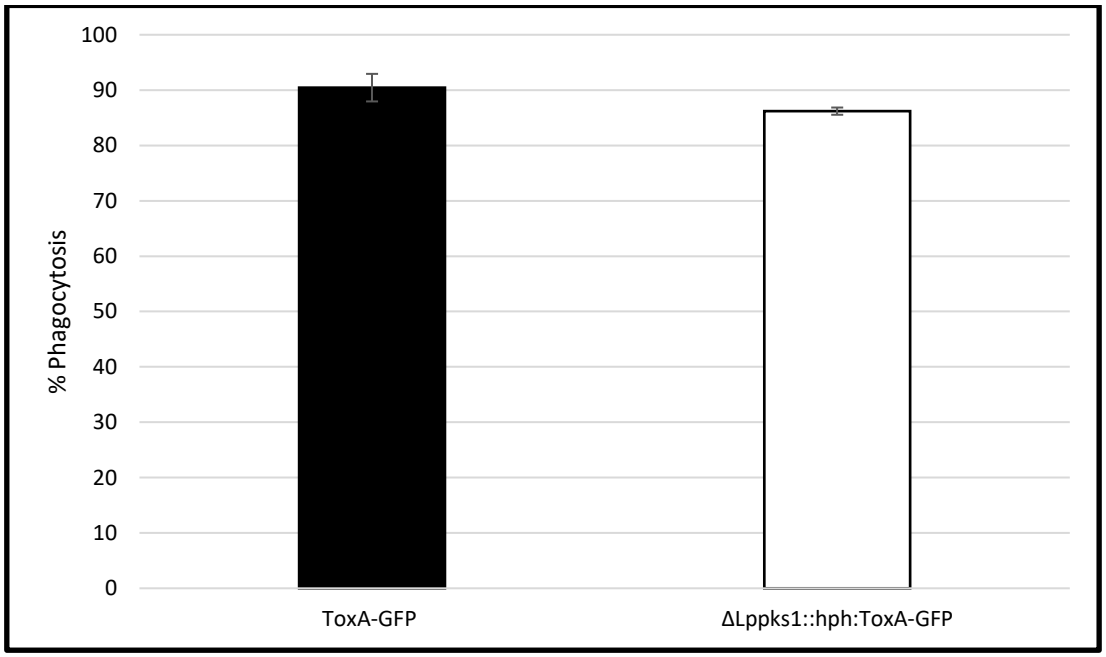

Figure 6.12: Percentage phagocytosis (P%) of *L. prolificans* strains. Spores were co-incubated with J774A.1 macrophage cells for 4 h. The results show no significant differences (*P*>0.05) in P% between the two strains. Each bar is the mean of three replicates ± standard error.

6.6 Phagocytic index (Pi)

Phagocytic index (Pi), defined as the average number of spores phagocytosed by each macrophage, was determined after co-incubation of macrophage with spores for 4 h. Results showed no significant differences in Pi values between the melanised strain ToxA-GFP and the non-melanised strain Δ*Lppks1*::*hph*:ToxA-GFP (Figure 6.13). The phagocytic index for strain ToxA-GFP was 3.6, and 3.2 for strain Δ*Lppks1*::*hph*:ToxA-GFP.

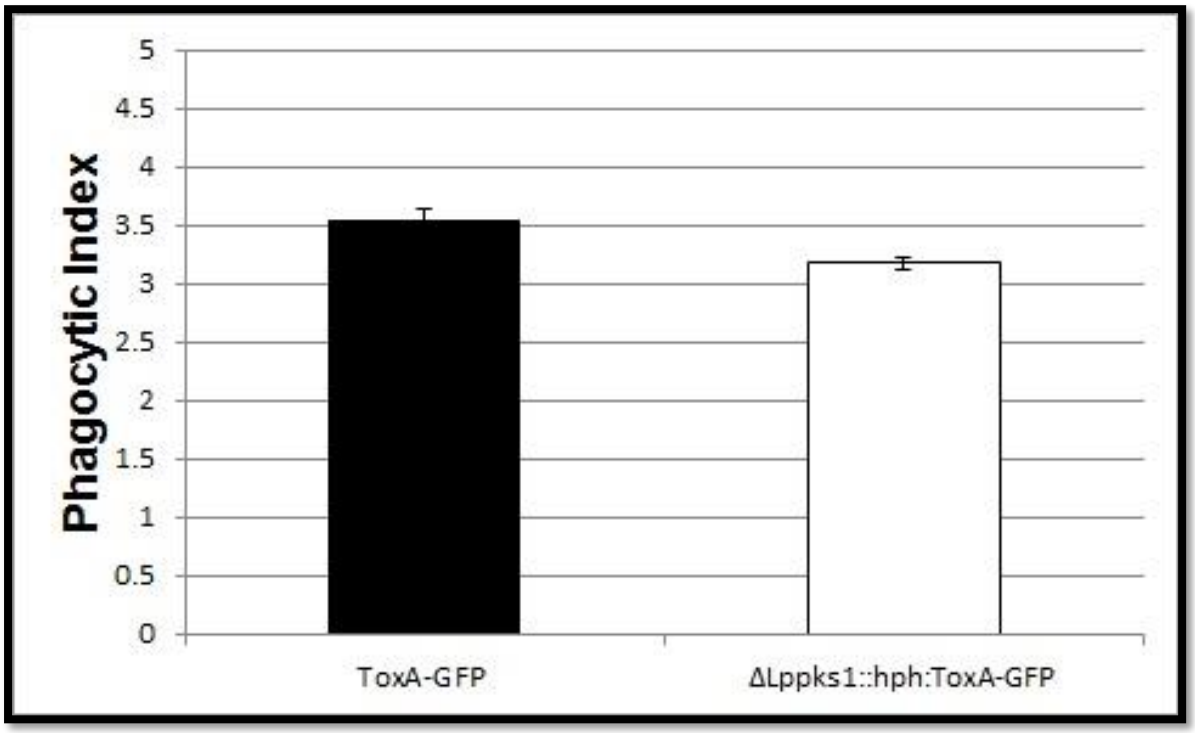

Figure 6.13: Phagocytic index of *L. prolificans* strains. No significant differences (*P*>0.05) in Pi values were found between ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP. Each bar is the mean of three replicates ± standard error.

6.7 Inhibition of spore germination by J774A.1 cells

Inhibition of spore germination was quantified after 6 h exposure of spores to J774A.1 cells to allow phagocytosis and germination of viable spores. Visualisation of germ tubes was aided by the use of the mutant strains expressing GFP (Figure 6.14). Results showed no significant differences in the ability of J774A.1 cells to inhibit conidial germination of melanised (ToxA-GFP) and non-melanised (Δ*Lppks1*::*hph*:ToxA-GFP) strains of the fungus. Percentage germination inhibition was 92.5±2.8 and 96.3±0.3 for ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP, respectively (Figure 6.15).

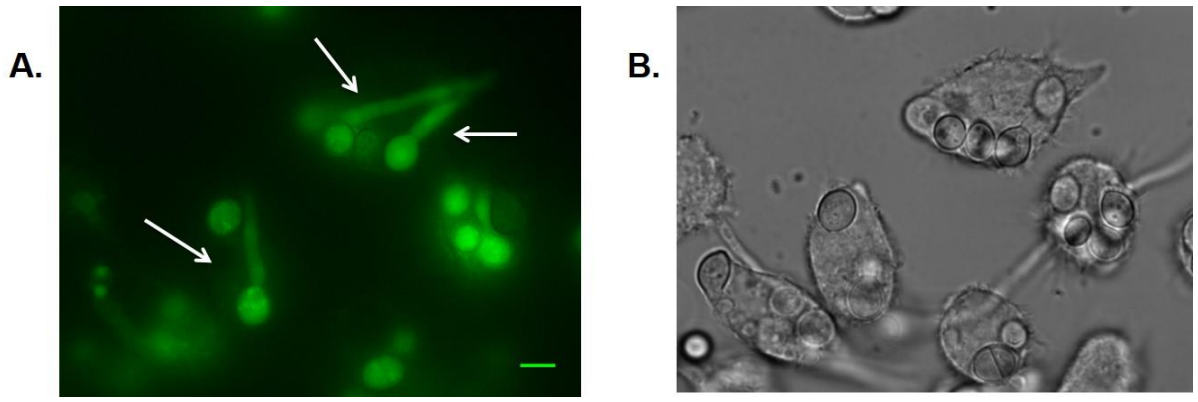

Figure 6.14: Atypical germination of *L. prolificans* conidia in J774A.1 macrophage cells. Spores and macrophage cells were co-incubated at 37 ^{o}C and 5% CO_2 for 6 h. **A)** Germinating spores of strain ToxA-GFP showing green fluorescence of conidia and germ tubes. **B)** Same image as A but examined under bright field microscopy. Scale bar =10 µm. While the majority of spores were prevented from germinating following phagocytosis, certain spores appeared able to avoid killing, and to produce germ tubes as shown.

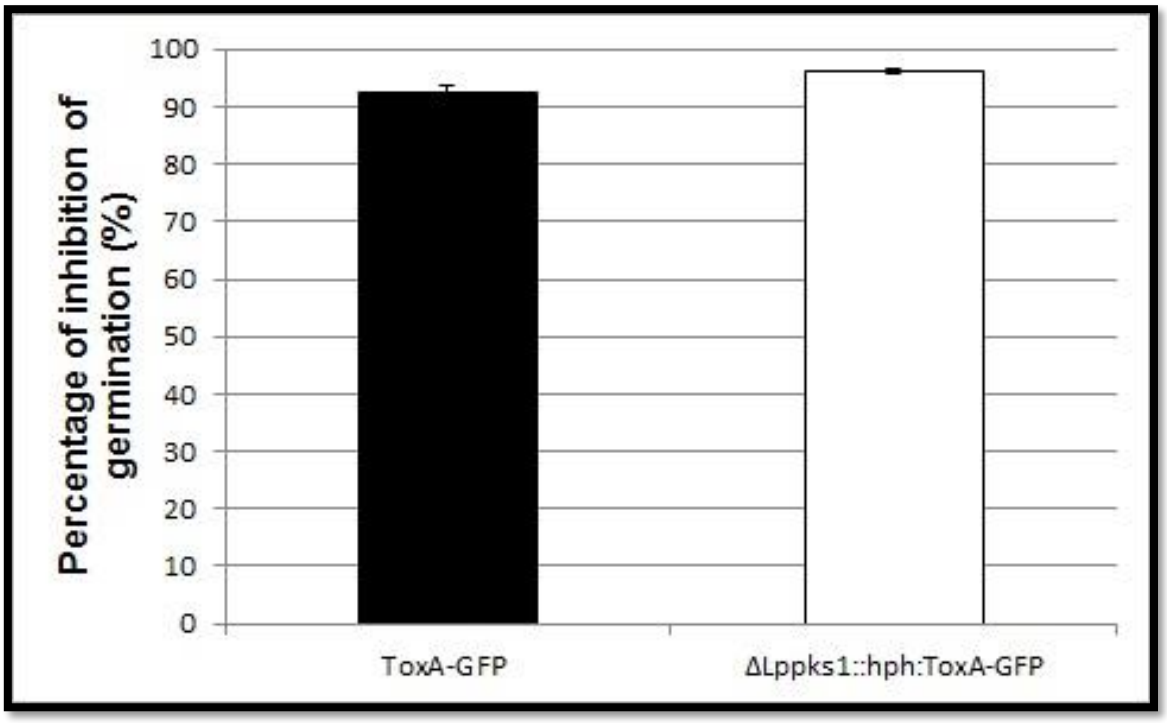

Figure 6.15: Percentage inhibition of germination of *L. prolificans* spores after phagocytosis. Conidia and macrophages were co-incubated at a ratio 1:10 at 37 ºC and 5% CO_2 for 6 h to allow phagocytosis and germination of spores inside J774A.1 cells. The results showed that no significant differences (P>0.05) in inhibition of germination percentages of spores from the melanised and non-melanised strains ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP, respectively. Each bar is the mean of 3 replicates ± standard error.

6.8 Determination of killing of *L. prolificans* spores by J774A.1 macrophages

6.8.1 Mycological culture

The ability of J744A.1 cells to kill phagocytosed spores of the melanised *L. prolificans* wild-type strain 3.1 and albino PKS-deficient strain Δ*Lppks1*::*hph* was determined by mycological culture of phagocytosed spores released from macrophages by cell lysis at different time periods of co-culture. The concentration of released spores was adjusted to 10^3 spores/ml, and the spores allowed to germinate and to develop into colonies on SDA. Using this procedure, no significant differences in colony-forming units (CFUs) were found between *L. prolificans* 3.1 and Δ*Lppks1*::*hph* at 6 h, 8 h, and 10 h (Figure 6.16). Nevertheless, the numbers of CFUs for both strains decreased over time, showing killing of *L. prolificans* spores by the macrophages.

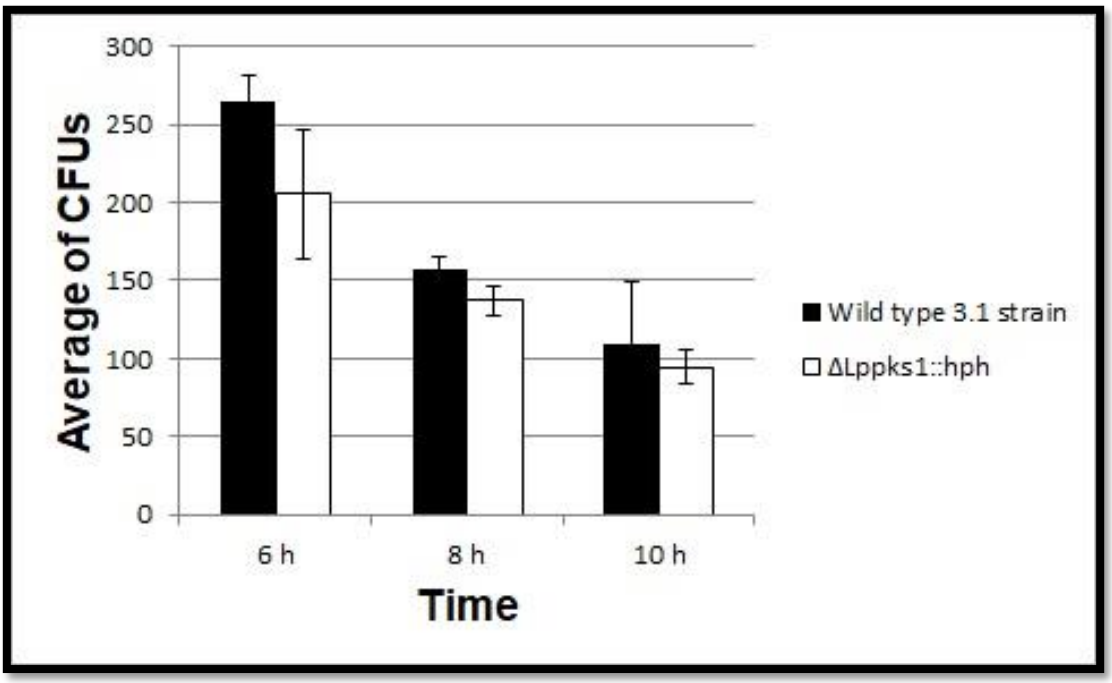

Figure 6.16: Viabilities of *L. prolificans* spores determined by mycological cultures. The number of colony-forming units (CFUs) of the two strains were determined 6 h, 8 h, and 10 h following co-culture of spores with J774A.1 cells. While the numbers of CFUs decreased over time, there were no significant differences (P<0.05) in CFUs for the two strains at each time point. Each bar is the mean of 3 replicates ± standard error.

6.8.2 MTT assay

The colorimetric MTT assay (Figure 6.18), which measures the metabolic activity of cells, was used to investigate the efficiency of J744A.1 cells in killing phagocytosed spores of *L. prolificans*. The percentage of spores killed by macrophages was determined using the relative absorbance values for spores incubated in the presence and absence of the phagocytic cells (see Section 6.2.5.2). It was hypothesised that melanin protects fungi from killing by macrophages. Macrophages were infected with melanised and non-melanised spores of *L. prolificans* strains at different time intervals (six, eight and eleven hours). Kill percentage was calculated using the formula in section 6.2.5.2. No significant difference between the kill percentage of the albino mutant Δ*Lppks1*::*hph* and the wild-type strain was observed after six hours of co-incubation (18.1% and 14.6% respectively). Similarly, the Δ*Lpscd*::*hph* and Δ*Lp4hnr*::*hph* mutants showed no significant difference to wild-type strain 3.1, with kill percentages of about 12.3% and 1.9% respectively. The efficiency of J744A.1 cells to kill the melanised wild-type strain 3.1 was greater (26.2%) after eight hours of co-incubation, compared to 2.3% with the albino Δ*Lppks1*::*hph* mutant. No significant difference was observed with the Δ*Lp4hnr*::*hph* and Δ*Lpscd*::*hph* mutants, where percentage of killing stood at about 24.2% and 13.8% respectively. Similarly, the complemented Δ*Lppks1*::*hph*:*PKS* strain of Δ*Lppks1*::*hph* restored the kill percentage to a similar level as with the wild-type strain 3.1 – about 20.5%. Similar results were obtained after eleven hours of co-incubation, when the percentage of killing (40.4%) was significantly greater in the wild-type strain 3.1 compared with 6.6% in the Δ*Lppks1*::*hph* mutant. The Δ*Lppks1*::*hph*:*PKS* complemented strain restored the kill percentage to about 41% – the same level as the wild-type strain 3.1. No difference was observed between the wild-type strain 3.1 and the Δ*Lpscd*::*hph* and Δ*Lp4hnr*::*hph* mutants, with kill percentages of about 35.7% and 22.5% respectively. All together, these results suggest that melanin plays no role in the protection of *L. prolificans* against the antifungal activities of the J744A.1 macrophage.

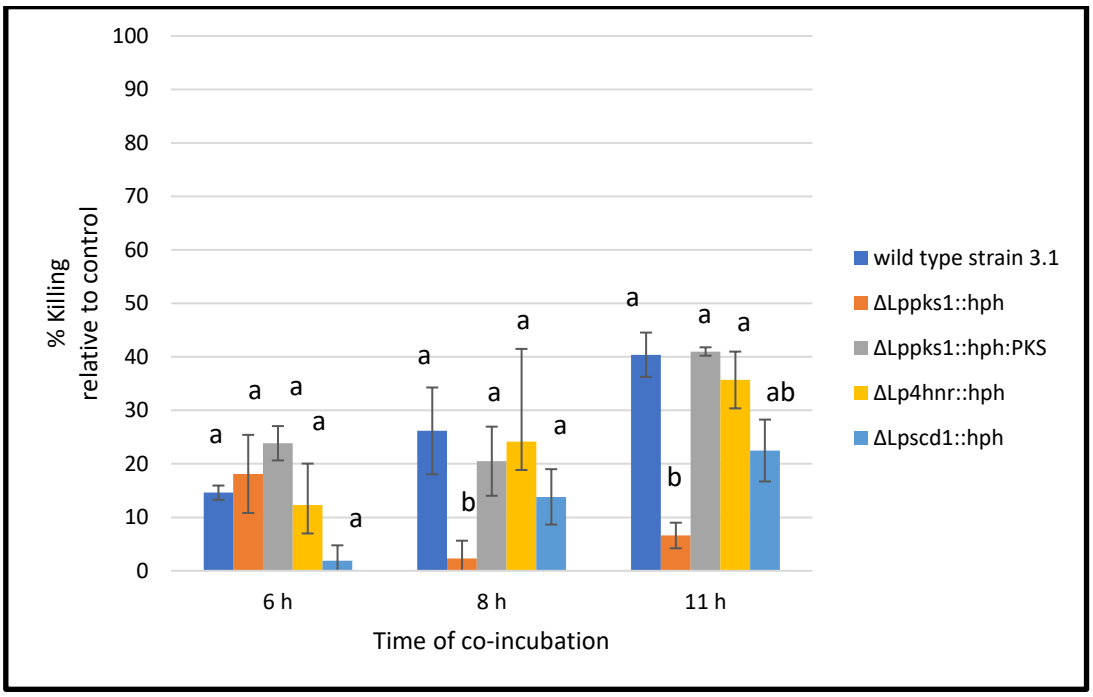

Figure 6.17. Antifungal activities of macrophage J744A.1 after six, eight and eleven hours of co-incubation with melanised and non-melanised strains of *L. prolificans*. An MTT assay was used to investigate the percentage of killing of the *L. prolificans* strain after incubation with J744A.1 cells. Each bar shows the mean of three replicates (± standard error) and similar letters represent no significant difference at $P>0.05$. Results suggest that there was no significant difference between the wild-type and other *L. prolificans* strains after six hours of co-incubation. The wild-type strain 3.1 showed greater sensitivity to killing by the macrophage than the albino Δ*Lppks1*::*hph* mutant after eight hours of co-incubation, while the complemented strain Δ*Lppks1*::*hph*:*PKS* restored the sensitivity to killing by the macrophage to similar levels as the wild-type strain. After eleven hours of incubation, a difference in survival rate was observed between wild-type strain 3.1 and the albino mutant, with the wild-type strain 3.1 showing greater sensitivity than the albino mutant. The complemented strain of the Δ*Lppks1*::*hph* mutant restored sensitivity to killing to a similar level as the wild-type strain 3.1. The Δ*Lp4hnr*::*hph* and Δ*Lpscd*::*hph* mutants showed no significant difference in kill percentage to the wild-type strain 3.1 at all time intervals.

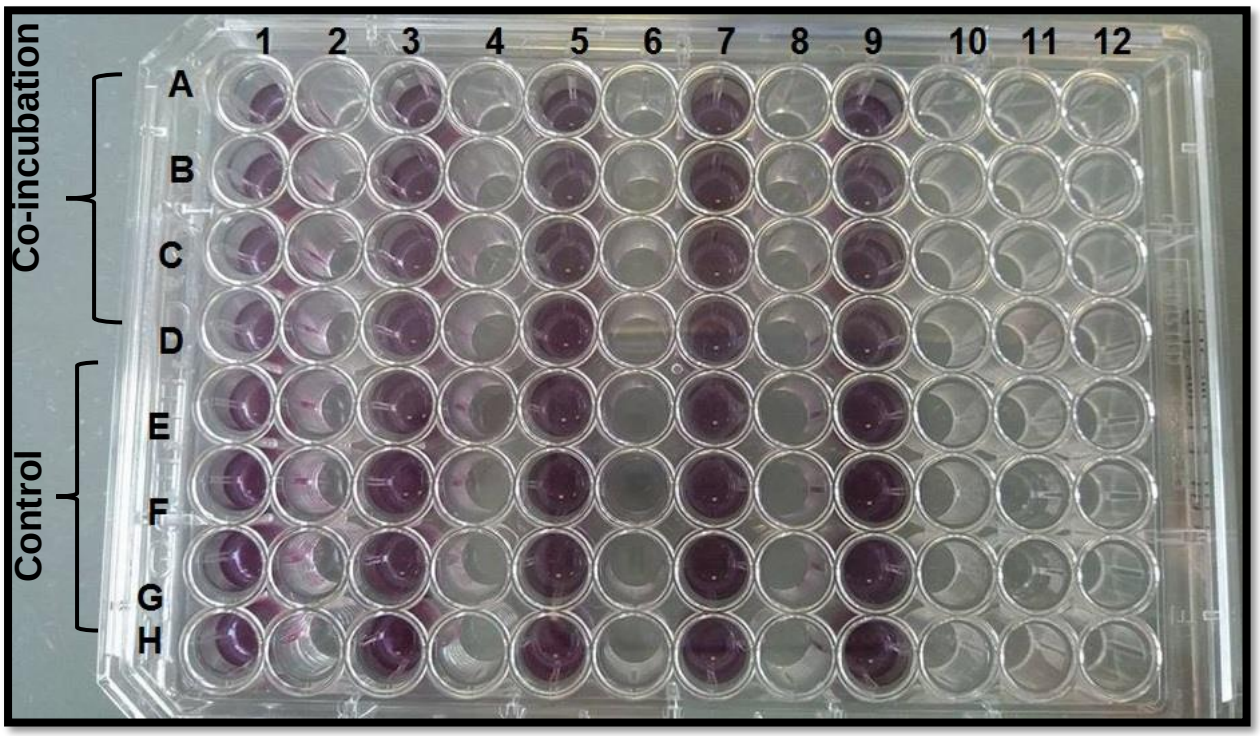

Figure 6.18: Image of a 96-well culture plate after the colorimetric MTT assay. Column 1 shows the colour reaction for the *L. prolificans* wild-type strain 3.1, column 3 is the albino mutant Δ*Lppks1*::*hph* mutant, column 5 is the complemented strain Δ*Lppks1*::*hph*:PKS*,* column 7 is Δ*Lp4hnr*::*hph*, and column 9 is Δ*Lpscd1*::*hph*. Rows A, B, C, and D (co-incubation), are the colour reactions for spores co-incubated with J744A.1 cells, while rows E, F, G, and H (control) are the colour reactions for spores incubated in the absence of macrophages. The absorbance values for the coloured products are used to determine the percentage of killing by using the formula in Section 6.2.5.2.

6.9 Pathogenicities of melanin mutants in *Galleria mellonella* (Wax moth)

To determine the optimum spore inoculum for pathogenicity assays, larvae were injected with a range of spore concentrations (10^3, $3x10^3$, $5x10^3$, 10^4, $3x10^4$, $5x10^4$, 10^5, $3x10^5$, $5x10^5$, and 10^6 spores/ml) for each of the fungal strains. The different spore concentrations gave the same rates of larval survival (based on numbers of melanised larvae post infection (Figure 6.19) for each of the strains, with the exception of 10^6 spores/ml. For this reason, pathogenicity assays were conducted with spore inocula containing 10^6 spores/ml.

At this inoculum concentration, larval survival over a 10 day period was greatest with the wild-type strain 3.1, followed by the scytalone dehydratase-deficient mutant Δ*Lpscd1*::*hph* (Figure 6.20). Lowest larval survival (or highest fungal pathogenicity) was found with the albino mutant Δ*Lppks1*::*hph*, while the Δ*Lppks1*::*hph* complemented strain Δ*Lppks1*::*hph*:PKS showed pathogenicity similar to that of the wild-type strain 3.1. Greatest pathogenicity was exhibited by the tetrahydroxynapthalene reductase-deficient mutant Δ*Lp4hnr*::*hph.* Survival of larvae injected with the GFP-expressing wild-type strain ToxA-GFP was sufficiently dissimilar to its non-fluorescent counterpart 3.1 to imply that random integration of the vector had altered its pathogenicity. Altered pathogenicity of the GFP-expressing mutant Δ*Lppks1*::*hph*:ToxA-GFP compared to its non-fluorescent Δ*Lppks1*::*hph* counterpart was also evident. Despite this, the dramatic increase in pathogenicity of the albino mutant and restoration of pathogenicity in the complemented mutant to levels similar to that of the wild-type strain 3.1, demonstrate a role for melanin in the pathogenicity of *L. prolificans* in vivo.

Figure 6.19: Response of *Galleria mellonella* larvae to infection with *L. prolificans*. The larva on the left shows characteristic melanisation as a result of infection by the pathogen. The larva on the right is healthy and unmelanised.

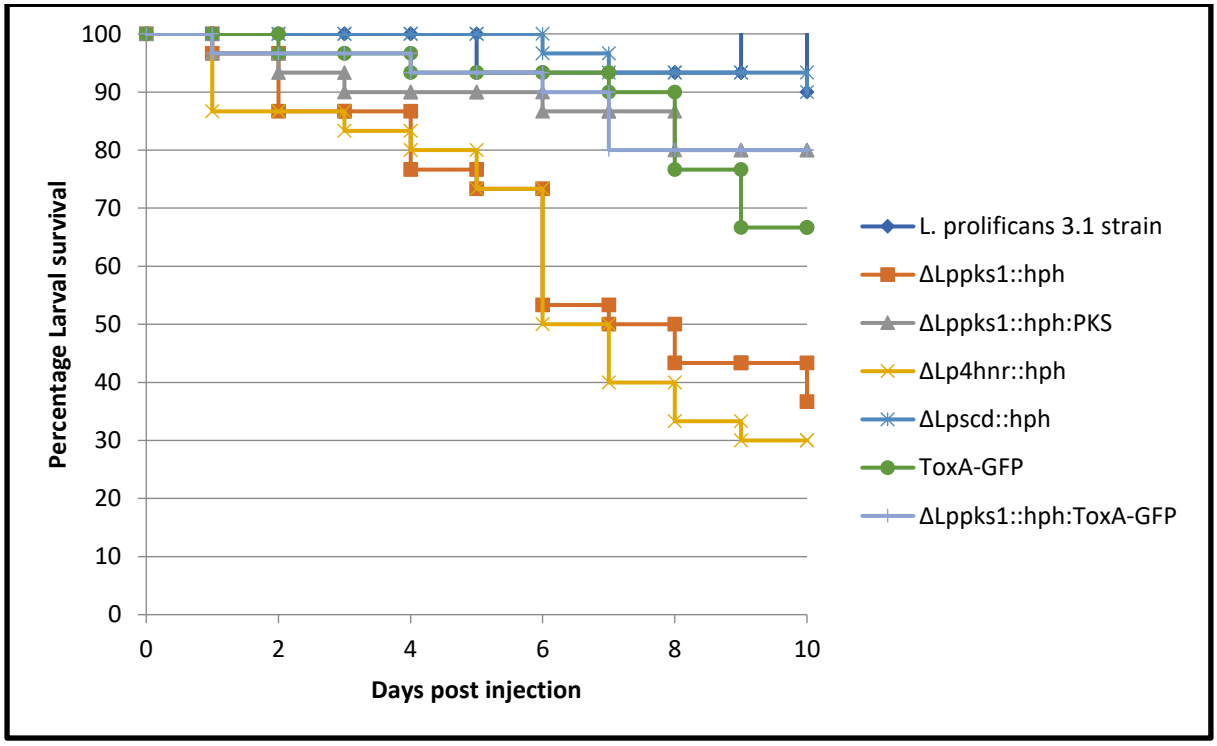

Figure 6.20: Kaplan-Meier survival curves for *Galleria mellonella* larvae injected with *L. prolificans* strains. The albino mutant Δ*Lppks1*::*hph* and mutant Δ*Lp4hnr*::*hph* show increased pathogenicity compared to the wild-type strain 3.1, while complementation of the albino mutant restores melanisation and gives a level of pathogenicity similar to 3.1. Ten wax moth larvae were injected for each strain and percentage survival of injected larvae monitored over a 10-day period. The experiment was repeated 3 times.

6.4 Discussion

The aim of this chapter was to investigate the protective role of melanin in *L. prolificans* against innate immune cell recognition and killing, and to determine its role in fungal pathogenicity. To achieve this, I studied macrophage J774A.1 phagocytosis and killing of melanised and non-melanised strains of the fungus, aided by the development of mutant strains expressing green fluorescent protein. I examined two methods for studying macrophage killing of phagocytosed spores *in vitro*, and used the invertebrate *Galleria melonella* (Wax moth) model to determine the role of melanin in the pathogenicity of *L. prolificans*.

Fungal species have developed several strategies to overcome host immune system defence, with melanisation considered to be one of the most effective mechanisms (Chai, *et al*., 2009). The shielding role of melanin against immune system recognition has been investigated previously in different dematiaceous fungi including *Aspergillus fumigatus, Cryptococcus neoformans, Wangiella dermatitidis, Paracoccidioides brasiliensis,* and *Fonsecaea pedrosoi* (Wang, *et al*., 1995; Schnitzler, *et al*., 1999 ; Cunha, *et al*., 2005; Volling, *et al*., 2011; Eisenman, *et al*., 2012). However, to the best of my knowledge, the work reported here is the first to investigate the role of melanin in immune cell evasion by *L. prolificans*.

Phagocytosis is an essential process in innate immune defence against a pathogenic fungus (Erwig, *et al*., 2016), which includes recognition of conidia, internalisation, and subsequent killing (Erwig, *et al*., 2016). I first investigate whether melanisation in *L. prolificans* influences spore uptake by J774.1 macrophages with the wild-type strain 3.1 and albino Δ*Lppks1*::*hph* mutant transformed with pCB1532 vector comprising the ToxA-GFP fragment for constitutive expression of green fluorescent protein (GFP). The GFP-expressing strains ToxA-GFP and Δ*Lppks1*::*hph*:ToxA-GFP showed no differences in pigmentation, hyphal growth and sporulation compared to their untransformed counterparts, indicating random integration of the GFP fragment with no discernable deleterious effects on gross morphology or melanisation.

There were no significant differences in P% (the proportion of macrophages with phagocytosed spores) or Pi (the average number of spores per macrophage) between GFP transformants of the wild-type strain and albino mutant suggesting that

macrophage recognition of *L. prolificans* occurs independently of fungal melanisation. This finding contradicts the observations of Chai *et al*. (2010), who argue that melanin shrouds fungal pathogen-associated molecular patterns (PAMPs), such as TLR-4 ligands and β-glucan on the surface of conidia, from recognition by host pattern recognition receptors (PRRs). The uptake of *Aspergillus fumigatus* conidia by epithelial cells is greater in a melanised strain compared to a melanin-deficient mutant (Amin, *et al*., 2014), while phagocytosis of non-melanised strains of the yeast *Cryptococcus neoformans* is greater than melanised strains, but can be increased by the capsule binding monoclonal antibody 2H1 (Wang, *et al*., 1995). Similarly, phagocytosis of non-melanised strains of the yeast *Paracoccidioides brasiliensis* by J774A.1 macrophage cells was greater than a melanised counterpart, even in the presence of complement proteins (da Silva, *et al*., 2006). As a negatively charged polymer, it was suggested that melanin interferes with cell wall charge, thereby inhibiting phagocytosis (Nosanchuk, *et al*., 2006). Despite these contradictory studies, my findings are consistent with those of Schnitzler *et al*. 1999, who showed no significant differences in recognition and phagocytosis of melanised and non-melanised strains of *Wangiella dermatitidis* by neutrophils.

Fungi have also evolved mechanisms to escape killing by phagocytic cells following internalisation (Chai , *et al*., 2010). For instance, it has been shown that *Histoplasma capsulatum* maintains the pH of the intra-phagosomal environment at 6 to 6.5, while *Candida albicans* forms germ tubes in the low pH of phagolysosomes enabling it to escape from phagocytes (Káposzta, *et al*., 1999; Newman, 1999). *Aspergillus fumigatus* similarly escapes phagocytes following spore germination and hyphal proliferation (Slesiona, *et al*., 2012). Gil-Lamaignere *et al*. (2001) compared the ability of monocyte-derived macrophages (MDM) to inhibit germination of *L. prolificans* and *Aspergillus fumigatus* conidia after ingestion. Their work suggests that germination of *L. prolificans* conidia is less pronounced than *Aspergillus fumigatus* (Gil-Lamaignere , *et al*., 2001) following ingestion, but nevertheless show that *L. prolificans* is able to evade killing by macrophages once internalised. My results confirm these previous

studies, demonstrating that the fungus is able to survive macrophage killing and to escape the phagocyte through spore germination and hyphal production.

Studying the fate of internalised fungal spores in macrophages is difficult, with different methods used to determine spore viabilities (colony forming units (CFU), microscopic detection of conidial germination, and flow cytometry (Marr, *et al*., 2001; Philippe, *et al*., 2003) often providing conflicting results. Each method has its limitations, and depends on the fungus in question. For example, CFU estimates are considered appropriate for yeasts, but not for filamentous fungi (Marr, *et al*., 2001). In this study, I used two methods to determine spore viabilities, the first used mycological culture to estimate CFUs, and the second using the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay to quantify metabolic activities of spores.

The MTT assay is a simple and precise method based on the ability of living cells to reduce and cleave the tetrazolium ring of the yellow MTT dye to a purple coloured formazan product using mitochondrial succinate-dehydrogenases (Mosmann, 1983). MTT is however toxic, and so a suitable concentration first needed to be established for use with *L. prolificans* spores. I determined this to be 0.125 mg/ml, a concentration that has been shown previously to not affect fungal viability (Meletiadis, *et al*., 2000). MTT assay was used to determine the viabilities of *Aspergillus parasiticus* and *Fusarium moniliforme* spores (López, *et al*., 2015). A limitation of the assay is that the metabolic activities of living organism differ, and so it is important to optimise spore concentration (Garn, et al., 1994) and incubation time (Meletiadis, *et al*., 2000) to maximise assay sensitivity. Previous work (Jahn , *et al*., 1995) has shown that incubation times can be reduced by adding menadione to the reaction mixture (Garn, *et al*., 1994; Jahn , *et al*., 1995). In this study, I used RPMI-1640 serum-free medium for spore-macrophage challenge assays since serum proteins precipitate from the culture medium, which interfere with the assay (Denizot, *et al*., 1986).

Using mycological culture for CFU estimates, no differences were found in the sensitivities of the melanised wild-type strain and albino PKS-deficient mutant. However, using the MTT assay, it was found that the spores of the melanised strain were more sensitive to killing by J774A.1 macrophage cells than the non-melanised mutant. This result suggests that melanin in *L. prolificans* may induce macrophage immune defence and that melanin does not provide complete protection from

phagocyte killing. Work conducted elsewhere has shown melanin to be an immunologically active particle (Alviano, *et al*., 2004), and injection of melanin into BALB/c mice induces an antibody response indicating the ability of immune system to recognise melanin as foreign antigen (Nosanchuk, *et al*., 1998). Furthermore, Mednick *et al*. (2005) suggest that melanin has immuno-modulatory effects, since melanised strains of the yeast *Cryptococcus neoformans* induce inflammatory responses *in vivo* with reduced survival in the host compared to melanin-deficient mutants (Mednick, *et al*., 2005). Melanin can also activate the alternative complement pathway, with deposition of C3 into melanin ghosts in BALB/c mice (Rosas, *et al*., 2002). Huffnagle *et al*. (1995) have shown that cryptococcal melanin cannot protect fungi from elimination by activated cells *in vivo* (Huffnagle, *et al*., 1995).

As mentioned previously, methods used to determine spore viabilities after phagocytosis can give conflicting results, and the two methods used here are no exception. Despite this, both methods suggest that melanin does not play a major role in protecting *L. prolificans* from killing by macrophages. Furthermore, a proportion of cells are able to evade killing, germinate and escape through production of hyphae. While it is thought that melanin may protect spores of certain fungi against fungicidal and fungistatic activities of macrophages (Wang, *et al*., 1995; da Silva, *et al*., 2006), the mechanism(s) by which *L. prolificans* evades killing have yet to be determined.

Larvae of the invertebrate *Galleria mellonella* (Wax moth) were used here to then investigate melanin as a determinant of pathogenicity in *L. prolificans*. A validated alternative to mammals for studying fungal pathogenesis (Kavanagh, *et al*., 2010), wax moth larvae are inexpensive to purchase and maintain, are less ethically contentious, and have innate immune systems that share many similarities with mammals (Kavanagh, *et al*., 2010; Thomaz, *et al*., 2013). Injection of the larvae is simple compared to injection of *Drosophila melanogaster* in the thorax, with larval injection through the pro-leg with *L. prolificans* spores (Lamaris, *et al*., 2007; Lackner, *et al*., 2015). I hypothesised that melanin would protect the fungus from larval immunity, leading to decreased pathogenicity of the albino PKS-deficient mutant. On the contrary, the albino mutant proved to be more pathogenic than its wild-type melanised counterpart, which was confirmed by restoration of pathogenicity similar to the wild-type strain in the PKS complemented mutant. This result suggests that melanin induces immune system defences, consistent with studies using melanised strains of

Cyptococcus neoformance and *Aspergillus fumigatus* in wax moth larvae (Jackson, *et al*., 2009; Eisenman, *et al*., 2014). The similar increase in pathogenicity of the tetrahydroxynaphthalene reductase mutant Δ*Lp4hnr::hph* also suggests that intermediates in the melanin-biosynthetic pathway (in this case the shunt product flaviolin) may act to increase pathogenicity. Jackson *et al*. (2009) noted a similar phenomenon with colour mutants of *Aspergillus fumigatus*, suggesting that an accumulation of toxic by-products by colour mutants may not lead to killing of wax moth larvae (Jackson, *et al*., 2009).

In conclusion, the work conducted in this chapter shows that melanin does not confer protection to phagocyte recognition and killing of *L. prolificans* spores by macrophage cells. Furthermore, increased pathogenicity of the albino PKS-deficient compared to the melanised wild-type strain 3.1, suggests shows that melanin is not a pathogenicity factor in this pathogen.

7. General Discussion

Fungi represent a serious threat to human health, with a recent report estimating up to 1.2 billion cases of fungal infection globally each year (Azevedo, *et al*., 2016). With the exception of certain endemic mycoses such as coccidiomycosis, fungal diseases are not notifiable, meaning that the true involvement of fungi in human disease mortality and morbidity is difficult to determine. Efforts have been made by organisation such as US Centres for Disease Control and Prevention (CDC) to calculate burdens, but in the absence of a concerted World Health Organisation (WHO) programme to monitor the global incidence of fungal diseases, the only measures are based on rough approximations (Brown, *et al*., 2012).

The reported increase in fungal diseases over the past few decades has coincided with increases in the numbers of immunocompromised individuals in the general population, and the emergence of Acquired Immune Deficiency Syndrome (AIDS), both at high-risk of infection due to immune system impairment. Ironically, the number of immunocompromised individuals has increased because of improvements in modern medicine, and the introduction of highly effective immunosuppressant therapies and aggressive anti-cancer treatments which, while improving rates of cancer survival and preventing transplant rejection, have increased the vulnerabilities of patients to opportunistic fungal pathogens (Samaranayake, *et al*., 2002; Richardson, 2005). At the same time, these opportunists have acquired resistance to antifungal drugs, while other inherently drug-resistant species have emerged as life-threatening pathogens of humans (Samaranayake, *et al*., 2002).

One such opportunist is *Lomentospora prolificans*, a highly virulent and drug-resistant pathogen that has emerged over recent years as a cause of life-threatening disseminated infections in immunocompromised individuals (Ortoneda , *et al*., 2002 ; Ruiz-Díez, *et al*., 2003). Recently, efforts have been made to identify the virulence factors of the pathogen (Cross, 2008), and mechanisms by which it resists anti-fungal drug treatments. As a dematiaceous fungus, melanin is a likely contributor to its survival in its natural habitats such as soil, and during host infection, since this cell wall polymer is known to protect fungi against environmental stresses (e.g. UV, oxidative stress), to alter anti-fungal drug susceptibilities, and to modify immune cell recognition and killing (Nosanchuk, *et al*., 2015). For these reasons, melanin has been studied

extensively as a survival mechanism of human pathogenic yeasts and moulds (Wang, *et al*., 1994a; Heinekamp, *et al*., 2012), but until now its contribution to the pathobiology of *L. prolificans* has not been investigated. For this reason, I set out to determine its role in the survival and pathogenicity of the pathogen. To do this, I used a molecular genetic approach to generate melanin-deficient mutants, by disrupting key enzymes involved in DHN-melanin biosynthesis.

The first two experimental chapters of this thesis (Chapters 3 and 4) identified two genes encoding enzymes involved in DHN-melanin biosynthesis; the polyketide synthase-encoding gene *PKS1*, and the scytalone dehydratase-encoding gene *SCD1*. Previous work in our group had used a targeted gene deletion approach to disrupt production of a third gene (*4HNR*), which encodes the enzyme 1,3,6,8-tetrahydroxynaphthalene reductase, generating a Δ*Lp4hnr*::*hph* mutant deficient in 4HNR production (Thornton, *et al*., 2015). This mutant had impaired pigmentation, abnormal hyphal growth and sporulation, and its cell wall integrity was damaged. These observations prompted us to expand our investigations into the contribution of melanin biosynthesis to *L. prolificans* survival by using targeted deletion of the *PKS1* and *SCD1* genes. It was hypothesized that deletion of these genes would generate mutants more sensitive to environmental stresses, to anti-fungal drugs, and to phagocytic killing by alveolar macrophages (the front-line effector cells of immunity and critical to the control of fungal infections). These suppositions were based on previous studies that had shown that non-melanised fungi are more sensitive to environmental stresses and immune cell killing than melanised fungi (Gessler, *et al*., 2014).

The natural habitats of *L. prolificans* have yet to be fully established, but it has been isolated from soil and from extreme environments (Thornton & Wills, 2015a). Indeed, the strain used in this thesis (strain 3.1) was isolated from estuarine sediments (Thornton *et al*., 2015b), a marine environment exposed to high salt concentrations, extreme fluctuations in temperature, anoxia, pollutants, ultra violet (UV) radiation and reactive oxygen species. From this perspective, *L. prolificans* can be regarded as a halophile, or extremophile, capable of withstanding harsh environmental insults where melanin might protect it from extreme conditions such as UV radiation, oxidative and thermal stresses, and salinity. In this study, I chose to examine the role of melanin in the survival of *L. prolificans* to UV exposure and oxidative stress. Many disease

processes of clinical interest involve oxidative stress, and damage by free radicals is an important component of immune cell killing of pathogens and their infectious propagules.

Investigating melanin as a protectant in *L. prolificans* might reveal strategies for its control. For example, UV radiation has been used to eradicate fungi in the indoor environment (Levetin, *et al*., 2001), with successful control of medically important species of bacteria and fungi (Boyce, 2016) through UVR exposure (Katara, *et al*., 2008). In Chapter 5, I showed that the albino PKS-deficient mutant Δ*Lppks1*::*hph* susceptible to UV radiation with significantly reduced spore survival compared to its melanised counterpart and the complemented strain. This shows that melanin plays a critical role in the survival of the pathogen to UV exposure.

In Chapter 5, I similarly showed that melanin plays an important role in the pathogen's resistance to oxidative stress. French *et al* (2004) previously demonstrated the effectiveness of hydrogen peroxide in eradicating pathogens, and H_2O_2 is commonly used as a disinfectant in the hospitals (Boyce, 2016). H_2O_2 also plays a pivotal role in immune system defence during killing in the phagolysosome of alveolar macrophages. However, dematiaceous organisms display resistance to oxidative damage by H_2O_2 and I have shown its importance in *L. prolificans* here also, where both the albino PKS-deficient mutant, and 4HNR-deficient mutant Δ*Lp4hnr*::*hph*, both displayed increased susceptibilities to oxidative killing.

Melanin has also been shown to diminish the effects of anti-fungal drugs, and has been shown to protect the pathogenic fungi *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Wangiella dermatitidis* from the anti-fungal drugs amphotericin B and caspofungin, but not the triazoles or flucytosine (van Duin, *et al*., 2002 ; Jr, *et al*., 2006). However, this does not appear to be the case with *L. prolificans*. In Chapter 5, I showed that the albino mutant Δ*Lppks1*::*hph* and the SCD-deficient mutant Δ*Lpscd1*::*hph* grow significantly better in the presence of amphotericin B than their melanised wild-type counterpart 3.1. Paradoxical growth of this sort has been observed in *Candida albicans* and *Aspergillus fumigatus* in response to high concentrations of caspofungin (Wiederhold, 2009), a drug that prevents cell wall β-1,3-glucan synthesis by inhibiting the enzyme β-1,3-synthase. This paradoxical growth in *Aspergillus fumigatus* is due to increased calcineurin-mediated biosynthesis of the cell wall polymer chitin (through

increased chitin synthase activity) that compensates for the loss of the β-1,3-glucan (van Duin, *et al*., 2002). A similar observation was made in *L. prolificans*, where exposure to the azole voriconazole resulted in an increased content of chitin in the cell wall (Rodríguez, *et al*., 2017). The results of my work suggest that melanin is not involved in resistance to the fungistatic drug amphotericin B. Indeed, the increased growth of the two mutants, especially the albino PKS-deficient mutant, in the presence of the drug suggest that intact melanin is required for amphotericin B efficacy, or that an as yet uncharacterised mechanism compensates for the loss of melanin biosynthesis, and protects the fungus from its fungistatic effects. While combinations of drugs are often used to treat *L. prolificans* infections (Bhat *et al*., 2008), my work intimates that the combined use of amphotericin B and melanin inhibitors should be avoided.

The protective role of melanin in *L. prolificans* against oxidative killing by H_2O_2 (Chapter 5), prompted me to investigate its involvement in susceptibility of spores to killing by alveolar macrophages (Chapter 6), and its possible contribution to the pathogen's virulence. The involvement of melanin in resistance to immune cell killing of fungi is contentious. While some authors highlight its protective role, shielding a pathogen from immune system recognition, others suggest that, as an immunologically active component of the cell wall (Allam, *et al*., 2012), it induces immune recognition and killing (Nosanchuk , *et al*., 2003 ; Eisenman, *et al*., 2012). In this study, I hypothesized that melanin shields the *L. prolificans* spore from recognition and subsequent killing by macrophages. However, results showed that melanin plays no role in the protection of the pathogen from immune cell recognition and killing by alveolar macrophages, with similar degrees of engulfment, and spore viabilities, of mutant and wild type strains after phagocytosis.

The contribution of melanin to the pathogenicity of the pathogen was investigated using the invertebrate infection model *Galleria mellonella*. As with the macrophage interaction studies, I proposed that melanin might act to protect the spores against the larval innate immune system, and that the melanin-deficient mutants would display reduced virulence. However, contrary to expectations, the albino PKS-deficient mutant was significantly more virulent than the melanised wild type strain, consistent with previous reports with *Cryptococcus neoformans* and *Aspergillus fumigatus* (Jackson, *et al*., 2009; Eisenman, *et al*., 2014). A number of possible explanations have been

put forward for this phenomenon. It has been suggested that intermediates in the melanin biosynthetic pathway that accumulate as shunt products are toxic to the larvae. Indeed, in the pathogenicity studies conducted here, the most virulent strain was the 4HNR-deficient mutant ∆*Lp4hnr*::*hph*. This mutant is unable to convert 1,3,6,8-THN to scytalone, resulting in the accumulation of flaviolin, a spontaneous oxidation product of 1,3,6,8-DHN, that is excreted as a reddish-brown pigment into the culture medium (Thornton, 2105b). The production of this red pigment has been reported in spores of mutants of *Aspergillus fumigatus* and *Wangiella dermatitidis* as a consequence of hydroxynaphthalene reductase disruption and flaviolin accumulation and has been reported previously to be toxic to plant (Wheeler, *et al*., 1985; Tsai, *et al*., 1999; Abou-Mansour, *et al*., 2004). The limited pathogenicity of the wild-type strain 3.1 in wax moth larvae is perplexing given the virulence of *L. prolificans* in mice (Elizondo-Zertuche *et al*., 2017), but is perhaps related to the induction of larval immunity by melanin. This would be consistent with the increased virulence of the albino mutant, and restoration to wild type levels in the complemented strain), in which no toxic intermediates are produced as a result of *pks1* deletion.

In conclusion, the results presented in this thesis show that melanin protects *L. prolificans* from UV radiation and from oxidative killing by H_2O_2, consistent with its survival in extreme environmental habitats. However, melanin was not found to play a role the resistance of the pathogen to the antifungal drug amphotericin B, or to protect the fungus from immune cell recognition or killing by alveolar macrophages.

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